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Original Article

Continuous assessment of concentrations of cytokines in experimental injuries of the extremity

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Abstract: *Background.* Inflammation plays an important part in the healing process. Little is known about the extent local inflammatory trauma response interacts with the central circulation and inflammation produced by central organs. The aim of the present study was to examine whether high cut-off microdialysis catheters offer potential to in real time assess interstitial cytokines variations in conjunction to markers of metabolism distal to a blunt vascular contusion. *Methods.* In a standardised contusion trauma model, microdialysis catheters (high MW (100kDa)) were inserted in the gracilis muscle distal to the trauma for the local assessment of IL-6, IL-8, TNF-α, total protein and the metabolic mediators (glycerol, puruvate and lactate). The contra lateral uninjured leg served as control of the centrally mediated inflammation propagated to the extremities. *Results.* The trauma led to a significant and quantitatively large (8-10 fold) increase in inflammatory cytokines (IL6 and 8) as measured both in the injured and control legs. There was only a minor, and not significant increase in concentrations of cytokines in the injured leg compared to the control leg.. There were no signs of ischemia in either leg. *Conclusion.* The new finding in this study is that both central, and local, inflammatory responses as well as metabolic mediators may be assessed continuously in skeletal muscle tissue distal to a major injury in an animal model. The findings suggest that the large trauma elicits a generalised inflammatory response to trauma rather than propagating a local one distal to the trauma.

Key words: Blunt trauma, microcirculation, inflammation, microdialysis, rat

Introduction

Inflammation plays a significant part in modern medicine, not least as a regular phenomenon in the normal healing process, but also because it has been claimed to negatively influence the course and outcome after critical illness where it has been correlated to the process of organ failure [1-5]. It has also been hypothesised that certain cytokines may be used to predict morbidity and outcome [2, 6].

Present knowledge about the inflammatory process is based mainly on studies that examined the effect of inflammatory mediators on the central circulation ([2, 4, 5, 7, 8]. Knowledge about local release and occurrence is, however, sparse, particularly when release over time after an intervention is examined [3]. The whole inflammatory process engages not only the central circulation but also other important areas such as the wound, where some of these mediators are produced; [3] other organ systems such as the lung may be important producers of the total inflammatory response even to distal injury. It has been hypothesised that effects of contusion release cytokines that are constitutively present from damaged cells and inflammatory cells in the vicinity of the trauma. Traumatized tissue may also be released into the circulation and transported to other areas and organs of the body where their antigenic properties are thought to trigger further production and release of cytokines [2, 6, 9]. The role of inflammatory cytokines in local microvascular dysfunction and the following systemic response after blunt vascular trauma is still un-

clear, probably because of the lack of an appropriate and valid model for *in vivo* detection. Microdialysis has been a promising one in studies of cytokine production, but reports have been sparse because there are inherent sample collection problems. Recent data however, have indicated that new high MW (100 kDa) catheters offer potential, when used together with dialysate containing a colloid, in real-time measurement of inflammatory mediators such as IL 6, IL 8 and TNF-α parallel to markers of metabolism such as glucose, lactate and puruvate [10]. This technique has, however, not previously been used to examine systemic compared to local inflammatory effects induced by contusion trauma.

The purpose of this study was therefore to evaluate the use of microdialysis with high MW (100 kDa) catheters, and to examine the interplay between local and systemic inflammatory trauma responses in skeletal muscle distal to a standardised vascular contusion [11, 12]. Local production of cytokines was analysed as were changes in muscular metabolism to relate local changes in inflammation to changes in microvascular flow and/or metabolism downstream of the given trauma. To differentiate between the locally produced trauma effects (cytokines/metabolic effects) and the systemic response, we compared data from the injured leg to that of the unaffected opposite control leg.

Material and methods

Animals

All experiments were approved by the local Committee of Laboratory Animal Ethics, and the animals were handled in accordance with the Principles of Laboratory Animal Care.

Sixteen male Sprague-Dawley rats, (mean body weight 350g) were used. The animals were anaesthetised with a mixture of ketamine (Ketalar, Parke Davies) 80mg/kg body weight and xylazine (Rompun, Bayer AG) 8mg/kg body weight given intraperitoneally. Anaesthesia was maintained with small doses of the same drugs given through an intraperitoneal catheter. Every hour 2.5ml of Ringer´s acetate was given intraperitoneally to compensate.

Trauma model

The trauma model has been described in detail elsewhere [11]. Briefly, after the anaesthetic, all animals were placed supine in a profile on a platform so that they were in as identical positions as possible. Each leg was held by a rubber band that was pinned to the platform. An aluminium tube, 60cm long, was attached to the platform and centred over the right femoral region. A close-fitting cylindrical weight that could fall freely through the tube without moving sideways was used. The lower end of the weight was circular, diameter 19 mm, and slightly rounded to cause a blunt impact. A cylindrical weight weighing 1250g and with a falling height of 50cm was used. That corresponded to a force of 2.16N/cm^2 when it hit the thigh. The trauma was directed directly towards the femoral vessels, which were easily palpated in the right thigh.

Microdialysis

A small skin incision was made over the inside of the rat's thigh in order to get good access to the gracilis muscle. The microdialysis catheter (CMA 60, CMA Microdialysis AB, Sweden) was inserted easily from its lateral border, and the full length was placed securely inside the middle part of the muscle. The catheters were perfused with dialysate (Ringer´s Dextran 60, Braun) at a flow rate of 0.3 µl/minute and an initial washout period of 30 minutes. This was done in order to establish a steady state, and dissipate any adverse reactions to the insertion of the catheter. The dialysate was then collected, in capped vials every 60 minutes for 3 hours. The vials were stored immediately in 4° C until analysis on the same or the following day. A CMA 600 was used for the analysis of lactate, puruvate, urea, glucose and glycerol concentrations.

IL-6, IL-8, and total protein were quantified using a commercially available enzyme-linked immunosorbent assay (ELISA).

Statistics

Data are presented as median, mean, and SD. Changes in the area of interest (dependent) were evaluated by a forward, stepwise multiple regression model using animal, leg (trauma/control) and time as independent variables (Statistica v. 7.0, Tuscon, USA). Probabilities of less than 0.05 were accepted as significant.

Results

All animals had severe macroscopic contusions of the femoral vascular bundle examined during exposure, at the end of each experiment, but there was no damage to the gracilis or its adjacent muscles. All microdialysis probes were found within the borders of the gracilis muscle.

Inflammatory mediators

Il-6 (pg/ml): Il-6 concentrations increased significantly (p<0.001) both in the control and the injured leg from 48.1 (28.5, 57.2) and 77.0 (55.1, 74.2) (mean, median and SD), respectively, to 1331.4 (974.9, 1271.9) and 2927.3 (1746.7, 4257.8), (**Figure 1**). There was no difference between the concentrations recorded in the control leg and those in to the injured leg at any of these times (p=0.5).

IL-8 (pg/ml): IL-8 concentrations increased significantly (p<0.001) both in the control and the injured leg from, 339.1 (222.0, 281.6) and 375.0 (356.3, 234.2) (mean, median and SD), respectively, to 2634.0 (1851.8, 1707.1) and 3372.3 (3051.5, 2260.4), (**Figure 2**). There was no difference between the concentrations recorded in the control leg and those in to the injured leg at any of these times (p=0.6).

TNF-α (pg/ml): TNF-α concentrations remained stable throughout the experiments both in the control and the injured leg (p<0.001.). TNF-α measurements in the control and injured legs varied from 3.2 (3.2, 0.4) and 3.2 (3.2, 0.4) (mean, median and SD), respectively, to 3.2 (3.2, 0.6) and 3.3 (3.2, 0.4), (**Figure 3**). There were no differences between the control and the injured legs.

Total protein (μg/ml): Total protein decreased significantly (p<0.001) both in the control as well as the injured leg from 594.5 (530.0, 312.9) and 589.1 (714.2, 186.1) (mean, median and SD) respectively to 420.6 (355.4, 174.5) and 438.9 (393.5, 197.8), (**Figure 4**). There were no differences between the concentrations recorded in the control leg and those in to the injured leg at any of these times (p=0.7).

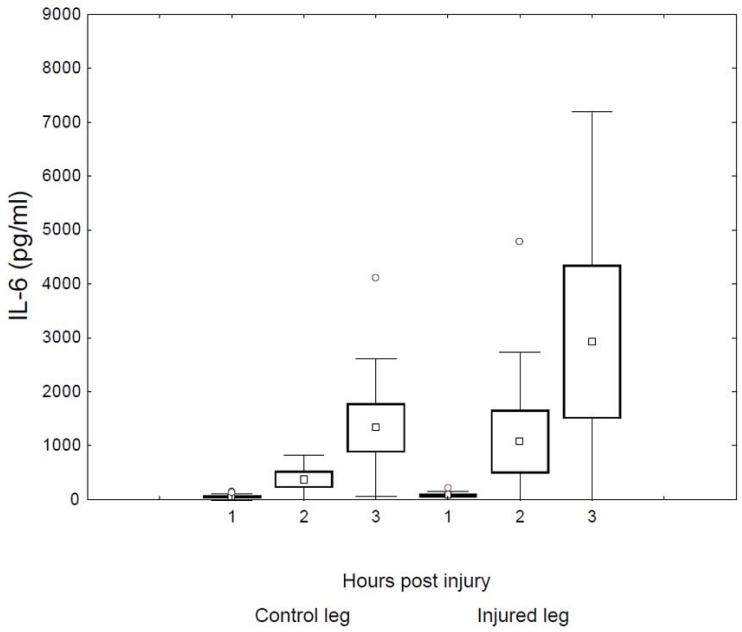

Figure 1. IL-6 changes in the control and trauma leg at 1, 2 and 3 hours post injury. Data as boxplot, (mean, box mean±SE; range mean ±SD and outliers). There was a significant increase over time (1,2 and 3h; p<0.001), but no differences between groups (trauma/control) were found.

Metabolic changes

Lactate (mmol/l): Lactate concentration increased significantly (p<0.001) both in the control and the injured leg from 1.197 (1.158,

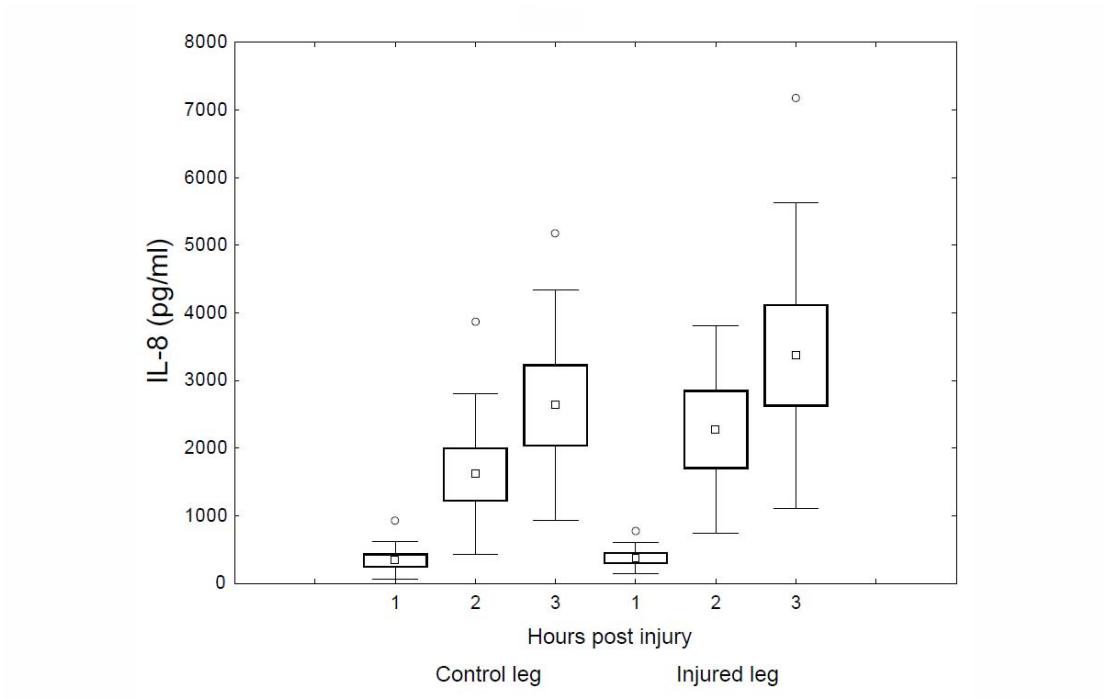

Figure 2. IL-8 changes in the control and trauma leg at 1, 2 and 3 hours post injury. Data as boxplot (mean, box mean±SE; range mean ±SD and outliers). There was a significant increase over time (1,2 and 3h; $p<0.001$), but no differences between groups (trauma/control) were found.

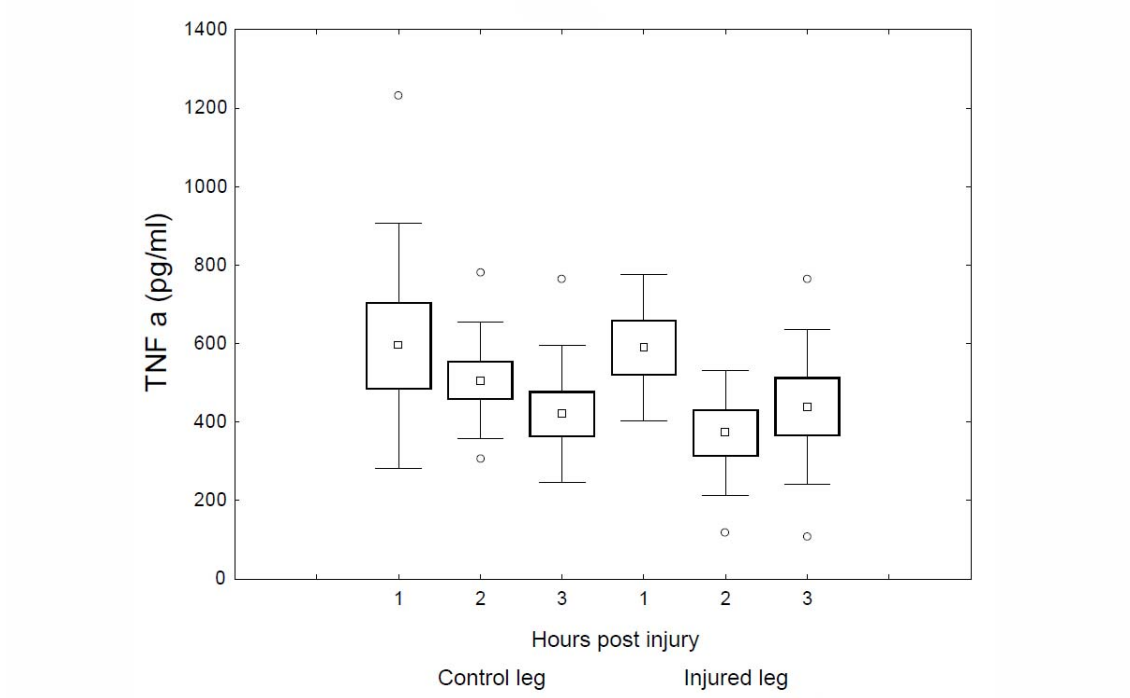

Figure 3. TNF-α changes in the control and trauma leg at 1, 2 and 3 hours post injury. Data as boxplot (mean, box mean±SE; range mean ±SD and outliers). No significant differences over time or between groups were found.

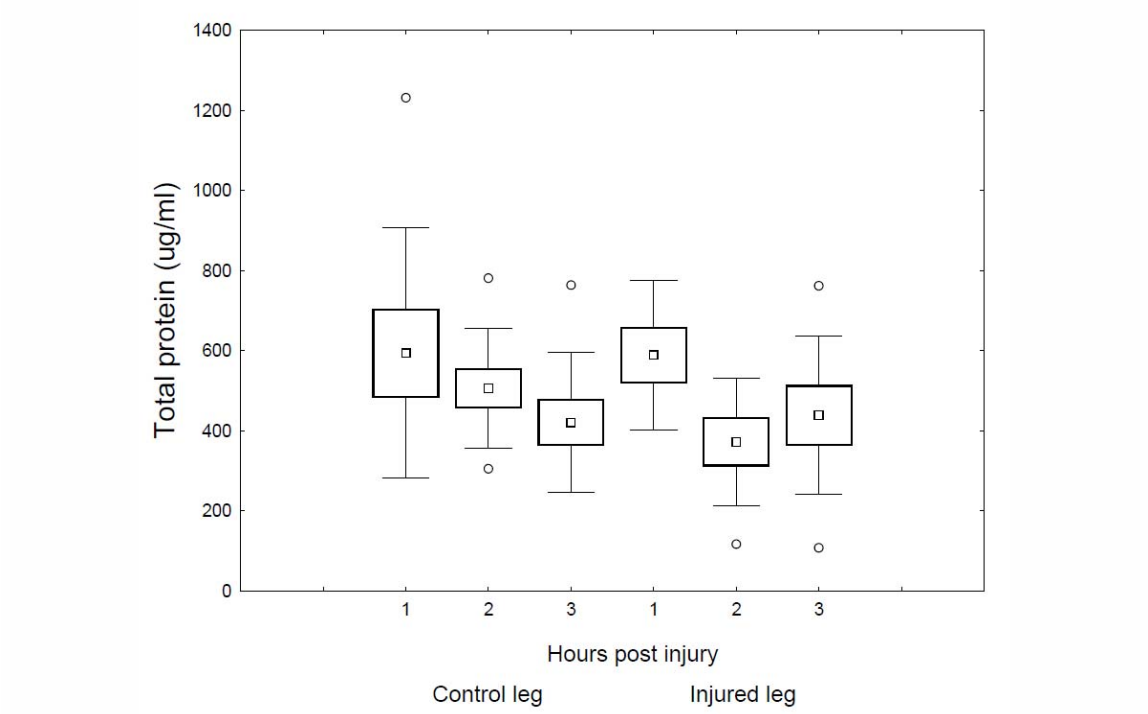

Figure 4. Total protein (µg/ml) changes in the control and trauma leg at 1, 2 and 3 hours post injury. Data as boxplot (mean, box mean±SE; range mean ±SD and outliers). No significant differences over time or between groups were found.

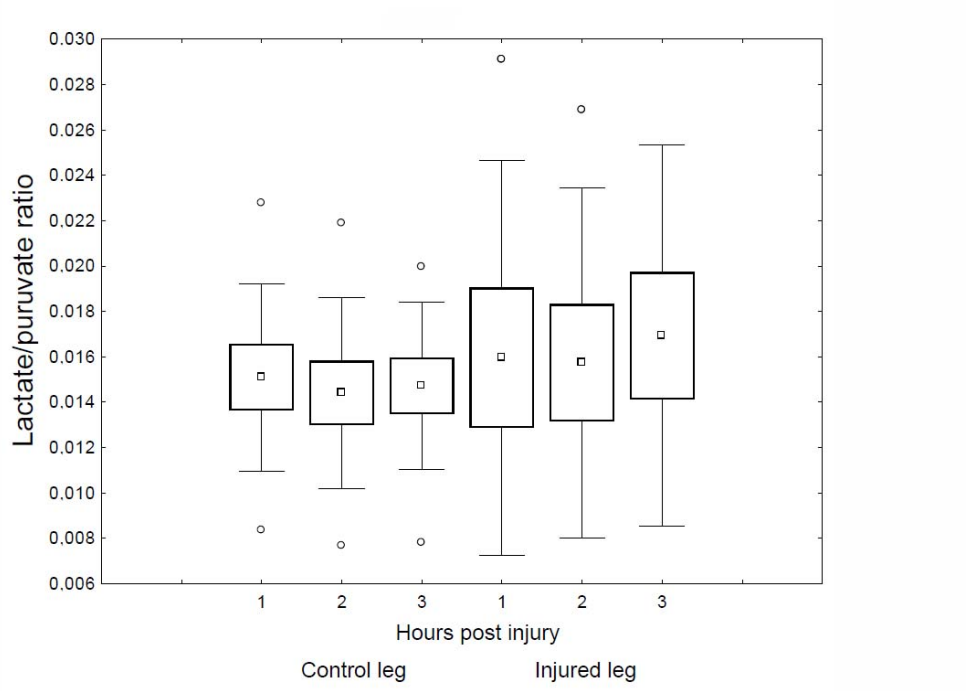

Figure 5. Lactate/puruvate quotient changes in the control and trauma leg at 1, 2 and 3 hours post injury. Data as boxplot (mean, box mean±SE; range mean ±SD and outliers). No significant differences over time or between groups were found.

0.484) and 1.454 (1.278, 0.569) (mean, median and SD), respectively, to 1.744 (1.576, 0.603) and 1.949 (1.782, 0.618). There were no differences between the concentrations recorded in the control leg and those in to the injured leg at any of these times.

Puruvate (mmol/l): Puruvate concentration increased significantly ($p<0.001$) both in the control and the injured leg from 82.8 (70.7, 34.2) and 104.0 (99. 7, 46.1) (mean, median and SD) respectively, to 130.6 (105.5, 73.7) and 148.3 (147.8, 96.1). There were no differences between the concentrations recorded in the control leg and those in to the injured leg at any of these times.

Lactate:puruvate ratio: There were no significant changes ($p=0.8$) in the lactate:puruvate ratio over time or between the control and injured leg, (**Figure 5**).

Glycerol (µmol/l): There were no significant changes ($p=0,4$) in either the control leg or the injured leg. Measurements varied between the control leg and the injured legs from 129.7 (104.9, 42.7) and 119.0 (108.9, 22.6) (mean, median and SD), respectively, to 152.0 (148.4, 43.5) and 144.9 (140.9, 26.6).

Discussion

In this study we aimed to evaluate a novel approach in microdialysis research in which interstitial markers of inflammation were registered in parallel with metabolic markers *in vivo*. The relation between cytokines and markers of metabolism, and their effect on microvascular dysfunction has up till now not been clear, probably because there were no appropriate and valid models for *in vivo* detection. Our results support our hypothesis that blunt vascular trauma causes a relatively small local response distal to the trauma, but a vast systemic inflammatory response that is e.g., propagated to the extremities. Our findings further support the use of microdialysis, with high cut 100kDa catheters, as a promising tool in the research of real time measurement of inflammation and metabolism in vivo.

High molecular weight cut-off membranes in microdialysis

Microdialysis is today well established and enables monitoring of metabolic wellbeing *in vivo* by real time measurement of metabolites such as glucose, lactate, and puruvate. It is also promising in studies of local cytokine production *in vivo* [13], however, reports have been sparse because of inherent problems in the collection of samples. New high MW (100kDa) catheters offer potential when used together with dialysate containing a colloid [10, 14]. Several studies have confirmed that Ringer´s dextran 60 balances the colloid osmotic pressure of the perfusate against the colloid osmotic pressure of the surrounding extracellular fluid, and gives nearby complete fluid recovery, which is a prerequisite for adequate interpretation of the results [10, 14]. This was taken into account and controlled in our study. Sample volumes obtained were measured and showed expected concentrations for the particular flow rate and time (data not shown).

Cytokines and blunt trauma

There is little knowledge about local cytokine response and its importance in the morbidity after blunt injury of the extremities or trauma directed towards the feeding vessels. Systemic effects elicited by trauma, or sepsis, or both, has been more widely explored and high systemic concentrations of IL-6 and IL-8 have an important role in the inflammatory response, and possibly in the development of multiple organ failure. The degree of cytokine release is thought to be relative to the total amount of tissue damage, and has therefore been referred to as “the antigenic load” of trauma [2, 6]. Real time interstitial measurements in the muscular compartment have not been made although they would probably help to explore reasons for damage to tissues distal to the trauma. Our regimen that included measurements in the opposite muscle enabled us to differentiate further between local and systemic effects of trauma. In the light of our results, it has been proposed that blunt trauma augments the release of IL-6 and 8 but not of TNF[15]. Various studies have failed to show increased concentrations of TNF [8, 16, 17] and it seems likely that our consistently low concentrations of TNF are because it is not an immediate inflammatory mediator after trauma, rather than a detection problem because of the microdialysis technique.

Trauma response

Microdialysis catheters were placed in muscle distal to the trauma and in the opposite mus-

cle (leg), for control, to record and differentiate between local and systemic effects of the injury. There was a trend, though not significant, which indicated a small local response to the trauma in both IL-6 and IL-8 (**Figure 1** and **2**). The systemic effect was shown as a more than 20-fold significant increase in IL-6, and 8-fold increase in IL-8 in the injured leg and in the control leg.

Measurements of lactate and puruvate showed significant increase secondary to trauma, although there was no significant difference between the injured leg and the control leg. The lactate/puruvate ratio remained stable throughout the experiments, confirming the lack of tissue ischemia. These data suggest that the significant, but relatively small, reduction in femoral and microvascular blood flow, that was shown in our previous studies in this experimental model[18], cause a relatively limited effect on the local blood flow and muscle metabolism. The rise in lactate and puruvate in both the injured leg and in the control leg illustrate the systemic response to the trauma.

Previous studies, using our standardised contusion model [11, 12], show that the femoral vessels, to a large extent, remain patent despite the extensive contusion injuries seen in the vessel wall. Alterations in microvascular blood flow and metabolism have been studied in skeletal muscle distal to the trauma [18, 19] to understand better the reason for the high tissue morbidity that is often seen after vascular contusion. A local inflammatory response with release of cytokines has been hypothesised to alter microvascular blood flow, as circulating cytokines such as IL-6 and IL-8 have been thought to have a key role in the pro-inflammatory response to blunt trauma [2, 3, 7, 15, 20]. TNF-α, on the other hand, seems to have an important role in the inflammatory response in septicaemia, but does not increase after blunt trauma [15]. Little however is known of the local production and the corresponding effects of these cytokines in the tissues and on the regulation of microvascular blood flow and tissue metabolism. Various tissues produce different concentrations of cytokines, and rat skeletal muscle cells also express mRNA for cytokine production after trauma [21], although increased transcription is unlikely to be of greater importance in the early course after tissue injury. Local release of constitutively present cytokines seems to be a more plausible cause of the rise in the interstitial muscle compartment. The initial local effect and subsequent systemic overflow of inflammatory mediators from the damaged cells at the trauma site may further induce systemic liberation of inflammatory mediators in more immune competent areas, giving rise to the systemic inflammatory response such as seen in our results.

Knowing that there is a high variability in cytokine production in various tissues, and high concentrations of cytokines are seen particularly in cancellous bone, and considerably less in skin and muscle [3], it is likely that the involvement of contusion with structures around the vascular bundle is of greater importance than the actual vascular trauma itself. When we combined our previous data with those of this study we found a 30% fracture rate of the femoral bone.

This is in line with previous reports of elevated concentrations of IL-6 and IL-8 after fractures of long bones and their correlation with the severity of the injury [2]. The relatively large spreads in absolute values are therefore; apart from inter individual alterations, most likely also to be dependent on the degree of bone damage produced by the trauma. It has, nevertheless, previously been shown that systemic increases in IL-6, but not IL-8, act as potent inhibitors of α-adrenergic contractility of smooth muscle in the vessel wall. Its action seems to be endothelium-independent and is not mediated through production of nitrous oxide. The effect of IL-6 on contractility of vessel walls is possibly one of the key mediators in the direct regulation of blood flow and blood pressure [22]. This may be a key factor in explaining our previous results that showed only a 20% decrease in femoral blood flow, and may also be of importance in changes on the microvascular bed secondary to vascular trauma.

Insertion effects caused by the catheter

Possible insertion trauma effects, due to the insertion of the microdialysis catheter into the muscle tissue, has been studied previously in human dermis and muscle [23, 24] as well as in the subcutaneous space in rats [25]. IL-6 concentrations detected in the subcutaneous space in rats and dermis in humans show a similar response to the insertion which is

quantitatively doubled compared to baseline values seen in human skeletal muscle. This difference confirms previous reports indicating that skin is immunologically more competent and contains significantly higher amounts of IL-6 and IL-8 compared with skeletal muscle [3].

IL-6 is in our study increased four times (1100pgml-1 compared to 500pgml-1) at 2 hours, compared to the corresponding situation in human skeletal muscle after probe insertion [23, 24]. Also when comparing our early increase, which we believe may be affected by the insertion trauma it is found that it is three times as high as the corresponding increase in the human dermis after probe insertion [23, 24] and more than tenfold when comparison is made with IL-8 in dermis after probe insertion [24]. Thus we believe the insertion effect in our model to be insignificant compared to the large response elicited by the contusion trauma.

Conclusion

The data in this study supports the use of high molecular weight cut off membranes in microdialysis for continuous assessment of both central and local metabolic mediators of the inflammatory response in skeletal muscle tissue. The findings suggest that the large trauma directed to the vascular bundle, muscle tissue and cancellous bone in the femoral region of the rat elicit a generalized inflammatory trauma response rather than propagating a local inflammation distal to the trauma.

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