Sialic Acid is a Marker of Lung Injury Following Lower Extremities Ischemia/Reperfusion

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Objective. This study tests whether sialic acid is a mediator of the lung injury following lower extremity ischemia/reperfusion (I/R).

Design. Prospective randomised study.

Materials and Methods. Thirty-one Sprague–Dawley rats were randomised into four groups: group 1, aorta was exposed but not clamped; group 2, aorta clamped for 3 h, followed by 1 h of reperfusion; group 3, 50 mg/kg pentoxifylline administrated before the aorta was clamped; and group 4, 1 mg/kg dexamethasone administrated before the aorta was clamped. Serial arterial blood samples for blood gas, tumor necrosis factor-α (TNF-α), and total SA (TSA) assay were obtained. The lungs were removed and histologically examined for evidence of injury.

Results. Groups 2, 3, and 4 had significantly higher peak serum TSA concentrations compared with groups 1 (group 1 vs. 2, p = 0.001; group 1 vs. 3, p = 0.002; group 1 vs. 4, p = 0.001). Group 3 had lower peak serum TSA concentration. Groups 2 and 4 had significantly higher peak serum TNF-α concentrations (p = 0.0001) compared with groups 1 and 3. Group 3 had lower peak serum TNF-α concentration. Lower TSA and TNF-α levels are associated with lesser degrees of lung injury.

Conclusions. TSA and TNF appear during events that lead to lung injury following lower extremity I/R.

Key Words: Sialic acid; Tumor necrosis factor-α; Torso ischemia and reperfusion; Lung injury.

Introduction

Acute ischemia and reperfusion of an extremity starts an inflammatory cascade, which eventually causes injury and damage of the extremity, as well as some degree of insult to distant target organs as a result of systemic response. Severe and sometimes fatal pulmonary complications secondary to this inflammatory process have been well described before.

Sialic acid (SA) is a member of an acetylated family of neurominidic acid that is widely scattered in mammalian organisms.¹ SA is a fragment of some glycoproteins and glycopeptides found in the structure of hormones and enzymes.¹² A close relationship has been described between SA and ischemic conditions in recent years.³⁴ Also, SA levels correlates with the acute phase reactants that appear in acute inflammatory reaction.⁵⁶ Tumor necrosis factor-α (TNF-α) causes pulmonary injury after hindlimb ischemia/reperfusion (I/R) model in rats.⁷⁹ Although the mechanisms have not been elucidated completely, recent works indicated that TNF-α is an essential component of the cascade of events that lead to I/R-induced lung injury.⁹¹⁰

The role of sialic acid in ischemia is not clear. The relation with systemic inflammatory response is clear and evident in previous works. Here in this presented study, we wanted to evaluate the correlation between serum TSA levels and lower limb ischemia. Lower limb ischemia and reperfusion is known to cause lung injury which is actually attributed to locally produced cytokines such as TNF-α. In our study design, we aimed to look for the changes in serum TSA levels, where the systemic inflammation is attenuated with drugs known to attenuate the systemic inflammatory response. This would enable us to decide whether TSA is a marker or a mediator in systemic inflammatory cascades that eventually result in distant organ damage, mainly to the lung. For this, we have used pentoxifylline and dexamethasone on this assumption. Since the mediator role of TNF-α in lung injury is known, this substance, with histopathological examination of the lungs were investigated for their...
correlative connection with TSA, if it was also a mediator.

Materials and Methods

An experimental animal study was conducted. Animal care complied with the ‘Principles of Laboratory Animal Care’ as formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985). All studies were approved by the Animal Care and Use Committee of Cumhuriyet State University Medical Faculty.

Model

The study conducted by Tassiopoulos et al. was referred to for the planning of the experiments. In short, the study protocol includes the cross clamping of the infrarenal aorta for 3 h followed by a 1 h reperfusion period, by removal of the cross-clamp, which forms a well characterized acute limb ischemia and reperfusion (I/R) model.

Experimental protocol

Thirty-one adult male Sprague–Dawley rats, weighing 380–460 g were fasted overnight. Intramuscular injections of ketamine (75 mg/kg) together with xylazine (5 mg/kg) were administered for sedation and anaesthesia. The animals were anaesthetized throughout the entire procedure with injections of one-fourth of the initial dose given every 20–30 min. A carotid arterial catheter was inserted for blood sample analysis, and a jugular venous line was established for intravenous fluid infusion through the same neck incision. The animals were given heparin of 1000 units/kg. The infrarenal aorta was exposed through a midline abdominal incision. Rats were randomized into four study groups:

- In group 1 (control), the aorta was exposed but not cross-clamped, and the animal was observed for 240 min (n = 7).
- In group 2, aorta was cross-clamped just above the bifurcation with special vascular clips for 3 h, followed by 60 min of reperfusion with the removal of the vascular clip (n = 8).
- In group 3, animals were pretreated with 50 mg/kg pentoxifylline intravenously, before aortic cross clamping (n = 9).
- In group 4, animals were pretreated with 1 mg/kg dexametasona intravenously, before aortic cross clamping (n = 7).

Animals in groups 3 and 4 were subjected to the same I/R time as the animal in group 2. Reperfusion was achieved with the removal of the vascular clip.

Arterial blood samples were obtained for total SA (TSA), TNF-α, and blood gas analysis. All the data were measured at baseline (prior to aortic clamping), after 90 and 180 min of ischemia, and 30 min after reperfusion in the study groups. In control group (group 1) baseline samples were taken when the aorta was exposed and at 90, 180 and 210 min after this. All the blood samples (0.5 ml) taken for studies were replaced with equal volumes of isotonic fluids to prevent acute volume depletion of the animals.

Sialic acid and TNF-α analysis

TSA assays were centrifuged immediately at 4000 rpm and serum was collected. These were stored at −20 °C until assayed. The serum TNF specimens collected after centrifugation were stored at −70 °C until they were analysed. An enzymatic assay was used for the assessment of serum TSA (Boehringer Mannheim). The results were analyzed spectrophotometrically and expressed as mg/dL. All TSA determinations were performed at the same time by an independent investigator who was blind to the study. The enzyme-linked immunosorbent assay (ELISA) was performed using the CytoScreen Rat TNF-α. Kit (BioSource, Camarillo, CA). The CytoScreen Rat TNF-α Kit is a solidphase sandwich ELISA. An antibody specific for TNF-α was coated onto the wells of the microtitre strips provided. Samples (including standards of known TNF-α content), control specimens and unknowns were pipetted into these wells. This was followed by the addition of a biotinylated second antibody. During the first incubation, the TNF-α antigen bound simultaneously to the immobilized antibody on one site and to the solution phase biotinylated antibody on a second site. After removal of the excess second antibody, the enzyme streptavidin-peroxidase is added. This bound to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all unbonded enzymes, a substrate solution was added, which was acted upon by the bound enzyme to produce colour. The intensity of this coloured product is directly proportional to the concentration of TNF-α present in the original specimen. The plates are read spectrophotometrically in a microplate reader at 450 nm and expressed as pg/ml.
The intra- and inter-assay coefficients of variation for the ELISA were 2.7% and 4.3%, respectively.

The animals were sacrificed by intravenous injection of pentobarbital sodium at the end of the experiment. The lungs were then removed and immersion-fixed in formalin. Fixed specimens were paraffin-embedded, sectioned in 4 μm pieces, and stained with routine hematoxylin-eosin stain. The same pathologists who were blind to the study examined the specimens. At least two different sections from each specimen were examined to determine the degree of injury accurately. Lung injury was recorded according to a semi quantitative score based on congestion, interstitial edema, PMN infiltration, and airspace hemorrhage. The scoring included: 0, no changes; 1, focal, mild, subtle changes; 2, multi focal mild and prominent changes; 3, extensive prominent changes. Although, this scoring system is subjective way of evaluation, two different and blinded pathologists performed the examination and scored on every specimen to overcome a possible bias.

**Statistical analysis**

All the measured values are expressed as mean ± standard deviation. In univariate analysis, Students t-test was used where appropriate (parametric continuous data) and p-values lower than 0.05 were accepted as significant. For the data where parametric criteria was not met, Mann–Whitney U test was applied. Due to multiple groups (more than two), Bonferroni corrections were made and Post Hoc tests (Tukey) were applied to determine the significantly different data. All these statistical analyses were carried out with commercially available software packages.

**Results**

Total sialic acid changes during the experiment for all groups are given in Table 1. When compared with baseline levels, after 90 as well as after 180 min of ischemia, there was a significant increase in all the groups except group 1. After 30 min of reperfusion TSA levels continued to increase in groups 2, 3, and 4, being significantly higher than the baseline, 90 min as well as 180 min levels. The differences were significant between groups 2 and 3, but were not significant between group 2 and 4 (Table 1).

After 90 min of ischemia tumor necrosis factor-α (TNF-α), levels had increased in all groups as compared to the baseline values. The increase was most pronounced in group 2 and 4 and the differences were significant when compared with groups 1 and 3 (Table 2). Groups 2, 3 and 4 had significantly higher levels of TNF-α when compared with group 1 (p = 0.0001). After 180 min of ischemia, TNF-α levels had decreased significantly in all study groups, but they were still significantly higher than the baseline levels. Again, TNF-α levels in group 2 and 4 were significantly higher than the TNF-α level in group 1 (p = 0.001 for group 1 vs. 2; p = 0.002 for group 1 vs. 4). After 30 min of reperfusion, TNF-α levels continued to decrease and almost returned to baseline levels. Values are presented as mean ± standard deviation.
decrease and returned to baseline levels in group 1 and 3. The difference in group 2 and group 4 between TNF-α levels were not different, but still these groups had higher levels when compared with group 1 and 3 (Table 2).

Throughout the experiments, pH remained unchanged in groups 1, 3, and 4. In contrast, in group 2 the pH decreased significantly during the reperfusion, indicating a degree of systemic metabolic acidosis (Fig. 1).

Histological evaluation revealed significant differences in the degree of lung injury between groups (Fig. 2). Groups 1 and 3 had lesions ranging from 0 to 1+, with average injury scores of 0.57, 0.88 respectively. Groups 2 and 4 exhibited extensive, prominent histological changes ranging from 1+ to 3+ and an average injury score of 2.62 and 2.42 ($p = 0.001$ for group 1 vs. 2; $p = 0.002$ for group 1 vs. 4; $p = 0.003$ for group 3 vs. 2; $p = 0.003$ for group 3 vs. 4).

Discussion

A correlation of plasma TSA levels and acute phase reactants in acute inflammations have been described, but the relation between ischemia and TSA is not clear. Ischemia of the lower extremities in a rat model caused significant increases in serum TSA levels in our study. As the ischemia continued, serum TSA rose as well. In our study groups, 90 min TSA levels were significantly higher compared to baseline levels and control group and reached higher levels at 180 min. This might be due to induction of systemic inflammation. Reperfusion of ischemic extremities also resulted with steady increases in TSA levels. Even though statistically non-significant, pentoxifylline seems to attenuate the rise of TSA during ischemia. This was not observed in dexamethasone pretreated group. TSA levels were slightly lower in group 3, compared to groups 2 and 4, after 3 h of hind limb ischemia. During the reperfusion period, a statistically significant difference was reached in group 3, compared to group 2 and group 4 (Table 1). Pentoxifylline is known to decrease the release of various cytokines, including TNF-α. Since TSA is a sensitive marker of inflammation, lower levels of TSA in group 3 can be due to pentoxifylline.

Lower extremity ischemia/reperfusion induces TNF-α production, but the exact cells responsible were not identified. In the work by Tassiopoulos et al., TNF-α levels reach their peak after 1 h and returned to baseline levels after 3 h. Even though the sampling times were different in this study, we observed significant increases at 90 min in groups 2, 3, and 4. The increase in control group was considerably lower, and was probably due to a response to surgical trauma. Major changes achieved in TNF-α levels with lower torso ischemia did not correlate with the duration of the ischemia in our study. Levels of TNF-α decreased significantly at 180 min and in the reperfusion period in all groups. We believe that TNF-α is one of the cytokines that is responsible for the induction of systemic inflammatory response, but a negative feedback might be responsible (which is still obscure to us) for the decrease after the acute phase. The rise of TSA did not correlate with blood TNF-α.
levels during the experimental protocol. Both of these molecules are probably indicators of the different events, but not directly responsible from the damage caused by ischemia or following reperfusion.

Pentoxifylline pretreated group showed lower increases of TNF-α, compared with group 2 and 4, but was still higher than group 1 at 90 and 180 min. Pentoxifylline increases c-AMP, which might be mechanism that lowers TNF-α levels. These actions of pentoxifylline may be due to its inhibitory effects on systemic inflammatory response. These effects were not achieved with dexamethasone in our study. Although contradicting with previous reports, anti-inflammatory effect of dexamethasone was not evident in our study which may be due to the dose used in our work. It could be stated that dexamethasone’s anti-inflammatory effects are evident if given after the inflammation takes place, instead of pretreatment. Pretreatment with dexamethasone did not prevent the lung injury after I/R without a logical explanation in our study.

TNF-α mediates lung injury by activating polymorphonuclear neutrophils and enhancing their sequestration to lung. Blockade of TNF-α release or activity can reduce neutrophil chemotaxis and sequestration, and attenuate the lung injury processes. In previous reports, administration of pentoxifylline or dexamethasone before ischemia was shown to inhibit the release of TNF-α and protect the lung against I/R induced injury. In the histopathological examination of lung tissue samples, the pentoxifylline pretreatment group had lesser injury when compared to groups 2 and 4. The rats with the highest SA and TNF-α levels in group 4 had the most severe lung injury despite the pretreatment with steroids. Lower TSA and TNF-α levels were associated with lesser degrees of lung injury, regardless of the pretreatment drug.

In lower limb ischemia, systemic acidosis is an expected finding as it occurred in group 2, but it is not clear why this was not evident in groups 3 and 4 (pretreatment groups). Tissue pH was not measured in our study. This finding helps us to speculate on the actions of dexamethasone. In previous works it is proven to attenuate systemic inflammatory response; it probably works on local tissue factors during ischemia. The steady pH in the dexamethasone group suggests its protective effects on local homeostasis in ischemic tissues, which may not be sufficient enough to attenuate the systemic inflammatory response. Many local factors are responsible for the initiation of systemic inflammatory reaction when a part of the body is under ischemic threat. These start a cascade that lead to lung injury. TSA and TNF-α are involved in these cascades. The results of our study suggest that TNF-α may be one of the mediators, but TSA is probably a marker of ongoing reactions or systemic metabolic changes. We can conclude that, as
expected, pentoxifylline pretreatment can attenuate the inflammatory process.

References


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