Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure

David Brealey, Sekhar Karyampudi, Thomas S. Jacques, Marco Novelli, Ray Stidwill, Val Taylor, Ryszard T. Smolenski, and Mervyn Singer. Mitochondrial dysfunction in a long-term rodent model of sepsis and organ failure. Am J Physiol Regul Integr Comp Physiol 286: R491–R497, 2004. First published November 6, 2003; 10.1152/ajpregu.00432.2003.—Although sepsis is the major cause of mortality and morbidity in the critically ill, precise mechanisms(s) causing multiorgan dysfunction remain unclear. Findings of impaired oxygen utilization in septic patients and animals implicate nitric oxide-mediated inhibition of the mitochondrial respiratory chain. We recently reported a relationship between skeletal muscle mitochondrial dysfunction, clinical severity, and poor outcome in patients with septic shock. We thus developed a long-term, fluid-resuscitated, fecal peritonitis model that reproduces many of the findings reported in septic patient skeletal muscle. We present findings from a 3-day rat model of fecal peritonitis that closely simulates the human disease process, combining physiological and biochemical markers of organ dysfunction (3). Although sepsis is the leading cause of mortality in the critically ill (14), its incidence is increasing; over 750,000 episodes are reported annually in the United States (2) where it is now the 10th commonest cause of death (20), carrying an estimated annual healthcare cost in excess of $17 billion.

Mechanisms by which sepsis leads to organ dysfunction remain to be established. Although microvascular flow abnormalities occur (21), findings of decreased oxygen consumption (24), elevated tissue oxygen tensions (4, 31), yet minimal cell death despite functional and biochemical derangements (19), suggest that the problem lies more in cellular oxygen utilization (and, perhaps, an ensuing organ shutdown) rather than a problem with oxygen delivery per se. As mitochondria utilize >90% of total body oxygen consumption to generate ATP, organ dysfunction could be a consequence of impaired bioenergetic processes. We recently demonstrated decreased respiratory chain complex I activity and lower ATP levels in skeletal muscle biopsies taken from critically ill patients in septic shock (7). These related to both severity of disease and eventual outcome. There was a further association with increased nitric oxide (NO) production and decreased levels of reduced glutathione (GSH), an important intramitochondrial antioxidant. These data support in vitro studies showing mitochondrial complex inhibition by reactive nitrogen species with a fall in ATP synthesis (8, 9, 35) and protection afforded by GSH (5). Whereas inhibition of complex IV activity is rapidly reversible (10, 12), there is a more persistent inhibition of complex I activity related to nitrosylation and perhaps nitration (3).

A number of important questions arise. What is the relevance of skeletal muscle data to “more vital” organs such as liver or kidney? Are these changes causal or epiphenomenal? Ethical and technical difficulties constrain the availability of vital human biopsy tissue, especially when repeated sampling is desirable to monitor disease progression and concurrent mitochondrial function. It is thus incumbent to develop representative animal models that reflect many, if not all, of the biochemical and physiological abnormalities evidenced in patients. However, as summarized in Ref. 33, laboratory models of sepsis show considerable variation in mitochondrial function and ultrastructural damage, due in no small part to the model itself.

We therefore sought to generate a long-term septic model that closely simulates the human disease process, combining physiological and biochemical markers of organ dysfunction with histology and markers of mitochondrial function. Such a model would enable monitoring of temporal changes, comparison of vital and less vital organs, and serve as a potentially useful test bed for therapies. We report findings from a 3-day rat model of fecal peritonitis that reproduces many of the findings reported in septic patient skeletal muscle.

EXPERIMENTAL PROCEDURES

Experiments were carried out in accordance with the Animals (Scientific Procedures) Act of 1986. Male Wistar rats (Charles River, Inc., 8 weeks old, weighing 250–280 g) were housed in a barrier system with the appropriate ventilation and temperature. All experiments were carried out in accordance with approved guidelines for the care and use of laboratory animals by the Health and Safety Laboratory, and an ethics committee of the School of Medicine and Dentistry. Animals were used in accordance with the Animal (Scientific Procedures) Act of 1986.

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Margate, Kent, UK), 250–275 g, were housed in the local animal unit for 5 days prior, with free access to food and water.

Instrumentation was performed under isoflurane anesthesia maintained via face mask. Femoral arterial and venous lines (internal diameter 0.28 mm, external diameter 0.61 mm) were inserted and tunneled subcutaneously to emerge at the nape of the neck. They were then mounted onto a swivel/tether system secured to the rat using four silk sutures. This enabled the rat, on recovery from anesthesia, to have unimpeded movement around its cage with free access to food and water. Both lines were flushed continuously with 0.15 ml/h heparinized n-saline (1:1000). Mean arterial blood pressure was measured (P23XL transducer, Viggo-Spectramed, Oxnard, CA) and recorded onto a precalibrated PowerLab system (ADInstruments, Castle Hill, NSW, Australia).

Twenty-four hours later, sepsis was induced by intraperitoneal injection of fecal slurry (0.625 ml/100 g body wt). This was prepared from the bowel contents of a rat from the same batch, suspended in n-saline and filtered to remove fibrous material. Sham-operated control animals received no injection to avoid accidental bowel perforation.

Fluid resuscitation was commenced 2 h later via the femoral venous cannula. For the first day, 20 ml/kg \(1^{-1}h^{-1}\) of a 1:1 solution of 6% hetastarch (Elohaes, Fresenius-Kabi, Runcorn, Cheshire, UK) and 5% glucose was infused. This was reduced to 17.5 ml/kg \(1^{-1}h^{-1}\) between 24 and 48 h and to 10 ml/kg \(1^{-1}h^{-1}\) between 48 and 72 h. Sham-operated control animals underwent the same operative procedures and fluid resuscitation regimen. Blood and tissue were also obtained from naive rats killed to obtain fecal contents.

Before death at predetermined time points, rats were scored as being mildly, moderately, or severely affected. The scoring assessed appearance, alertness, and blood pressure (Table 1). The rat needed to show at least two characteristics within appearance and alertness categories to obtain a score for that category. The blood pressure reading was used providing the line was patent and the trace was stable for 10 min before death.

Animals were reanesthetized either before or at 4, 24, 48, and 72 h after induction of sepsis. Skeletal muscle (hindlimb) and liver samples were obtained and snap-frozen into liquid nitrogen. Cardiac puncture enabled blood sampling for measurement of pH, gases, and base excess. Animals were then killed by decapitation, and organs were obtained and snap-frozen into liquid nitrogen. Cardiac puncture enabled blood sampling for measurement of pH, gases, and base excess. Animals were then killed by decapitation, and organs were obtained and snap-frozen into liquid nitrogen.

Mitochondrial complex activities, nitrite/nitrate levels, GSH, adenine nucleotide, and muscle phosphocreatine and creatine concentrations were assessed using well-described spectrophotometric and reverse-phase HPLC techniques (11, 15, 17, 26, 29, 34). Briefly, complex activities were measured at 30°C in a Uvikon XS spectrophotometer (Secomam, Domont, France) and expressed as a ratio to citrate synthase activity as this mitochondrial matrix enzyme is resistant to oxidative attack. Complex I activity was measured as the rotenone-sensitive decrease in NADH at 340 nM, complex II/III activity as the succinate-dependent antimycin A-sensitive reduction of cytochrome c at 550 nM, and complex IV activity as the disappearance of reduced cytochrome c at 550 nM. GSH was measured by HPLC with electrochemical detection while nucleotides, phosphocreatine, and creatine were measured by HPLC with UV detection (Hewlett-Packard 1100 Chromatography System, Palo Alto, CA). Nitrite/nitrate levels were determined using a modified Griess method; this assay depended on the enzymatic reduction of nitrate to nitrite, which was then converted into a colored azo compound detected spectrophotometrically at 540 nM.

Data are presented as means ± SE. Overall comparisons over time and severity were made using two-way ANOVA using SPSS 11.0 software (SPSS, Chicago, IL). Comparisons within each time point were made by one-way ANOVA and post hoc least-squares difference testing.

RESULTS

Clinical Outcomes

Two hundred rats were successfully instrumented and made uneventful postoperative recoveries. All rats appeared clinically well at time 0 (i.e., on injection of fecal slurry). Fifty-seven rats (body wt 299 ± 2.2 g) were used as sham-operated controls while sepsis was induced in 143 rats (303 ± 1.5 g). A further nine rats comprised the naive, noninstrumented, nonanesthetized group.

The sham-operated group became mildly edematous with occasional piloerection but otherwise looked outwardly normal, continuing to eat and drink and maintaining an interest in their environment. All septic animals displayed clinical manifestations of ill health such as hunched appearance, piloerection, bloating, and a loss of interest in their environment; the severity of which allowed clinical grading into mild, moderate, and severe (Table 1).

Continuous blood pressure data collection was achieved in 52 septic and 25 sham-operated control rats over the full duration of each study. In the remainder the arterial line occluded due to its fine caliber. The fall in mean blood pressure was related to the severity of illness (\(P < 0.00001\)), becoming statistically significant in the moderate and severe groups (compared with controls) by 24 h, and in all septic groups by 48 h (Fig. 1).

At 4 h it was not possible to categorize the septic rats clinically. By 24 h, 57% of rats were classified as mildly septic, 17% as moderate, and 26% as severe. At 48 h, 34% were classified as mild, 41% moderate, and 24% severe. At 72 h 73% of the rats still alive were classified as mild and 27% as moderate. None of the severely affected rats were alive at 72 h. The protocol demanded death at fixed time points; however, 39 rats died beforehand, giving a nondetermined mortality of 27.3%. Three of these animals died before 24 h, 31 between 24 and 48 h, and five between 48 and 72 h. Pilot studies showed that no severely affected animal (whose MAP remained consistently <60 mmHg) survived beyond 6 h of the classification being made. Assuming that all severely septic animals would have died by 72 h but that no mild or moderate group animals

Table 1. Severity scoring system

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
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<tbody>
<tr>
<td>Hunched</td>
<td>Hunched</td>
<td>Marked piloerection</td>
<td>Markedly bloated</td>
</tr>
<tr>
<td>Piloerection</td>
<td>Marked piloerection</td>
<td>Markedly bloated abdomen</td>
<td>Conjunctival injection</td>
</tr>
<tr>
<td>No bloating</td>
<td>Sunken eyes</td>
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<tr>
<td></td>
<td>Sunken eyes</td>
<td></td>
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<tr>
<td>Alertness</td>
<td>Depressed level</td>
<td>Markedly (or absent)</td>
<td></td>
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<tr>
<td></td>
<td>of alertness</td>
<td>depressed level</td>
<td></td>
</tr>
<tr>
<td>Occasional</td>
<td>Little interest in</td>
<td>No interest in</td>
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<td>interest in</td>
<td>environment</td>
<td>environment</td>
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<tr>
<td>environment</td>
<td></td>
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<tr>
<td>Moves freely</td>
<td>Moves with difficulty</td>
<td>No movement</td>
<td></td>
</tr>
<tr>
<td>Mean blood</td>
<td>&gt;90 mmHg</td>
<td>75–90 mmHg</td>
<td>&lt;75 mmHg</td>
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<td>pressure</td>
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levels in the severely affected rats at both 24 and 48 h. Serum albumin levels, reflecting the balance between hepatic production and increased microvascular leakage, were also significantly lower in the sicker rats at 24 h. The severely affected rats had significantly lower base deficits and pH values ($P > 0.0001$) from blood taken by cardiac puncture compared with the other groups (data not shown).

**Histology**

Histology was either unremarkable or showed only mild or focal abnormalities. Neither apoptosis nor necrosis was a major feature.

The serosal surfaces of the intestines showed an acute inflammatory reaction consistent with fecal peritonitis. The severity of inflammation was roughly proportional to the severity of clinical disease noted in individual rats. The intestinal mucosa and submucosa showed no signs of significant inflammation. Renal histology showed no evidence of either tubular necrosis or tubular obstruction. The majority of the livers was normal or contained only scattered foci of mild chronic inflammation. A few animals developed capsular abscesses consistent with the associated peritonitis. Sections of the lung were unremarkable in many of the animals. However, some contained small, scattered foci of acute inflammation, which in some cases was associated with perivasculary acute inflammation. The latter was associated with small thrombi in a minority of cases. The pleura and pericardium were unremarkable in the majority of animals; however, a few had foci of acute pleural or pericardial inflammation. Skeletal and cardiac muscle appeared unremarkable. Representative histology from organs of sham-operated and severely septic animals is shown in Fig. 2.

**Mitochondrial Function**

**Complex activities.** Complex II/III activity remained unchanged over time and with sepsis in both skeletal muscle ($P = 0.2$) and liver ($P = 0.8$) (data not shown).

Complex I, II/III, and IV activities were higher in naive liver compared with skeletal muscle. Of note, there was a progressive rise in hepatic complex I activity in sham-operated animals over time ($P < 0.0003$) but a fall in complex IV activity, reaching a nadir at 24–48 h ($P < 0.002$) (Fig. 3). Skeletal muscle complex activities in the sham-operated animals remained unchanged throughout.

Both skeletal muscle and liver complex I activity fell with increasing disease severity in the septic rats, with significantly higher activities in the sham-operated controls.
lower values in the severely affected animals at 24 (liver, muscle) and 48 h (muscle). On the other hand, there was a trend for liver and muscle complex IV to rise compared with time-matched sham-operated controls, albeit only being statistically significant at 24 h in the muscle of mild (P = 0.006) and moderately (P = 0.0003) affected rats.

Nucleotide levels. In the naive rats, ATP levels were higher in skeletal muscle compared with liver (Fig. 4). Hepatic ATP concentrations were significantly lower in the sicker rats at 24 and 72 h compared with both sham-operated controls (P < 0.05) and mildly septic rats (P < 0.05). In skeletal muscle ATP levels were higher in mild and moderately septic rats compared with sham-operated rats at 24 h (P < 0.01) but lower in the moderately septic rats at 72 h (P = 0.04).

AMP levels showed the converse changes to those described above for ATP (Fig. 4). Hepatic ADP levels rose in the septic animals, increasing with disease severity, albeit only statistically significant at 48 h (P = 0.05). Likewise, skeletal muscle ADP levels were significantly higher in mild (P = 0.007) and moderately septic rats (P = 0.03) at 24 h.

Hepatic ATP:ADP ratio was lower in severely affected rats at 24 h (compared with mildly septic controls, P = 0.02) and in moderately affected rats (significant at 48 and 72 h, P = 0.001). The total adenine pool did not vary with either time or sepsis (Fig. 4).

The skeletal muscle ATP:ADP ratio was significantly lower in septic rats at 4 h, and at 72 h in the moderately septic rats (P = 0.02 and 0.0002, respectively). The total skeletal muscle adenine pool was lower in the sham-operated rats at 24 h compared with mild (P = 0.003) and moderately septic (P = 0.006) and moderately affected rats at 72 h (P = 0.006) (Fig. 4). Although the skeletal muscle phosphocreatine:creatine ratio did not change significantly over time, it did show a downward trend with increasing sepsis severity (data not shown).

Tissue Nitrite/Nitrate and GSH Levels

A severity-dependent (and equivalent) rise in tissue nitrite/nitrate levels was seen in both liver and skeletal muscle of the septic rats between 24 and 48 h. By 72 h, these levels had returned to sham-operated control values (Fig. 5).

GSH concentrations were ~10-fold higher in liver compared with muscle at all time points. In septic animals GSH levels showed severity-dependent falls in both liver and muscle, which persisted at 72 h (Fig. 5).

DISCUSSION

Sepsis-associated multiorgan dysfunction is the predominant cause of mortality in the critically ill. Although the precise pathophysiology remains elusive, there is evidence to suggest...
a pivotal role for a bioenergetic abnormality. This is supported by our recent patient study that made the original observation of a significant association between disease severity, outcome, and mitochondrial dysfunction (7). The additional findings of increased NO production and decreased protection from GSH implicate these molecules although do not prove causality.

Here we present the results of a long-term (3 day) rat model of sepsis that was designed to simulate the human condition. In terms of insult, subsequent fluid resuscitation, biochemical abnormalities, histological findings, mitochondrial dysfunction, evidence of tissue NO production and GSH depletion, and subsequent mortality, we do feel a broadly representative model of human sepsis has been achieved. Antibiotics were not used while the relatively short time course in these young, healthy rats, with maximal deterioration at 24–48 h, more closely resembles the rapid deterioration and recovery often witnessed in children compared with the more prolonged illness frequently seen in the elderly. An important feature of this model is the aggressive volume resuscitation given to compensate for the decreased oral intake, vasodilatation, increased capillary leak, and third-space losses occurring in sepsis. Considerable effort was made in pilot studies to determine a satisfactory yet not excessive level of volume loading to avoid confounding our results with the effects of tissue hypoxia from underresuscitation and persisting hypovolemia.

This model of fecal peritonitis produced a spectrum of disease from mild to severe, despite using a standardized protocol on an outwardly homogeneous population of similar size and age. Although the bacterial load in the fecal slurry will have differed, there was still a marked variation in clinical response despite the same slurry being used in up to six animals from the same litter at the same time. This highlights the potential utility of this model to examine genotypic and phenotypic differences. Although the course of an individual

Fig. 4. ATP, AMP, ATP:ADP ratio and total adenine pool analyzed at the predetermined time points. Open bars represent sham-operated controls, crosshatched bars represent 4 h septics, light gray bars represent mildly affected, dark gray bars represent moderately affected, and black bars represent severely septic rats. *Significant difference ($P < 0.05$) from sham-operated control at same timepoint.

Fig. 5. Nitrite/nitrate concentration ([NOx]) and reduced glutathione concentration ([GSH]) analyzed at the predetermined time points. Open bars represent sham-operated controls, crosshatched bar represents 4 h septics, light gray bars represent mildly affected, dark gray bars represent moderately affected, white bars represent severely septic rats. *Significant difference ($P < 0.05$) from sham-operated control.
animal was unpredictable, the clinical classification of mild, moderate, or severe produced a comparable level of organ dysfunction with respect to raised levels of urea and creatinine (renal dysfunction), alkaline phosphatase (liver), and decreased albumin (liver, capillary leak). Mortality is likely to be related to cardiovascular failure as the degree of renal, hepatic, or pulmonary impairment, detected either biochemically and/or histologically, was not severe enough to suggest direct causation. Persistent hypotension despite aggressive volume resuscitation was the usual event preceding death.

The key findings of progressive mitochondrial dysfunction related to disease severity and NO production, with improvement on resolution of the illness, were reflected in both vital (liver) and nonvital (skeletal muscle) tissues. This is extremely pertinent to translational studies in patients in whom skeletal muscle is more readily and safely accessible. Significantly higher levels of GSH and mitochondrial complex activities and lower ATP levels were found in liver compared with skeletal muscle. This represents the different metabolic roles of the two tissues, possibly exaggerated in sedentary laboratory animals, as the liver has a persistently high background rate while muscles require only occasional bursts of metabolic activity. The ATP levels we found in rat skeletal muscle are approximately threefold higher than those found in humans, using identical methodology and equipment (7). This may suggest important species differences in nucleotide metabolism. However, differences in fiber types or different content of extracellular matrix can also contribute to this difference.

Although the ATP concentration is a good measure of cellular energetic status, the ATP:ADP ratio is perhaps a better reflection of the energy available for metabolic processes as this ratio is mainly influenced by the balance between cellular energy supply and demand. The lack of change in this ratio in muscle compared with liver may reflect the additional energy store provided by phosphocreatine.

The reduction in both liver and muscle ATP and energy charge seen in the sicker animals, together with the concurrent rise in AMP (suggestive of increased ATP hydrolysis), point to a decrease in ATP production and/or an increase in ATP utilization. We speculate that ATP utilization is more likely to decrease in an attempt to offset the fall in ATP production, thereby preventing a fall in ATP to a critical threshold sufficient to trigger apoptotic or necrotic cell death pathways. This would support the histological absence of cell death in the multiple organs examined. ATP turnover would be an ideal, although practically taxing, measurement to address this question.

Compared with the sham-operated controls, severely septic rats had hepatic and muscle complex I activities that were 20–22% lower at 24 h. This finding is similar to the difference in complex I activity found between septic and control human patients (16% difference in all septic patients and 30% in the nonsurvivors) and similar to other diseases known to be associated with mitochondrial dysfunction such as Parkinson’s disease (32). Inhibition of complex I by either NO or its metabolite, peroxynitrite, is via a reversible yet longer-acting nitrosylation of thiol groups in the complex, or by irreversible nitration (3, 6, 30).

The increased (or unchanged) complex IV activities seen in this study again reflects our patient data. NO is known to rapidly and reversibly inhibit complex IV activity by competitive binding with oxygen at cytochrome oxidase (10). Free NO levels remaining in the homogenates are unlikely to be high enough to observe this reversible inhibition, especially at the much higher Po2 (room air) under which the assay is performed. The observed rise in muscle complex IV activity may be due to an NO-mediated increase in the transcription of complex IV protein (25).

Although rodent production of NO in sepsis is much greater than in humans (28), only relatively modest (2- to 3-fold) increases in nitrite/nitrate production were seen in both muscle and liver, peaking at 24–48 h and returning to sham-operated levels by 72 h. These data mirror the pattern of clinical response; although cytokine levels were not measured in this study, they do suggest resolution of the acute inflammatory response. NO is also heavily implicated in the cardiovascular failure of sepsis, causing vascular hyporeactivity, vasodilatation, and myocardial depression (36). The temporal relationship of tissue nitrate/nitrate levels to blood pressure is consistent with this and with the notion of death being a primarily cardiovascular event.

Sepsis was also associated with a fall in tissue GSH concentrations, which correlated with clinical severity. As previously demonstrated by our patient study (7) and by cell models (5), the fall in GSH was associated with lower complex I activity and increased NO production. GSH is an important intramitochondrial antioxidant, detoxifying peroxynitrite and hydrogen peroxide and maintaining protein thiol groups in a reduced state. The lower GSH levels may have arisen from an increased rate of oxidation to oxidized glutathione (GSSG) and/or a decrease in the de novo synthesis of GSH. An increase in oxidant production is well recognized in sepsis, and this may overwhelm antioxidant defenses (13). It may also result in a decrease in activity of GSSG reductase, the enzyme that reduces GSSG back to GSH (1, 23, 27). A loss of the GSH precursor glutamine is also seen in sepsis, and thus GSH synthesis may be limited (16). The persistently depressed levels of GSH at 72 h, despite the apparent resolution of the acute inflammatory process, may suggest that one of these latter mechanisms may be important.

Despite biochemical multiorgan dysfunction and evidence of inflammation (more so in lung and liver compared with kidney and muscle), the lack of gross histological damage and cell death is an intriguing and underrecognized characteristic of sepsis-induced organ failure. This finding mirrors those reported by Hotchkiss et al. (19) on postmortem samples taken from septic patients who died of multorgan failure. It may be postulated that in the face of a prolonged and systemic inflammatory insult, with overproduction of cytokines, oxygen, and nitrogen reactive species and other mediators, and associated hypoperfusion and tissue hypoxia, that the organ responds by switching off its energy-consuming biophysical processes. This hibernation/estivation response could be viewed as a last-ditch attempt to prevent irreversible cell damage and allow eventual recovery of normal function, particularly in those organs such as the kidney whose cells are poorly regenerative. Such a phenomenon is well recognized in ischemic heart disease (18); it is highly plausible that similar mechanisms may be operating in sepsis.

In conclusion, we report a resuscitated long-term rat model of sepsis that has many similarities to the human condition. The model manifests evidence of bioenergetic abnormalities
that correlate with the severity of the septic response and mortality. Improvement in mitochondrial function was associated with resolution of clinical features and recovery of biochemical function. The temporal relationship with increased NO production, plus decreased protection afforded by GSH depletion, implicates NO as an important factor in the underlying pathophysiology. The many similar findings in muscle and liver suggest that skeletal muscle can be used as a surrogate for vital organs. We acknowledge that these findings may all be epiphenomenal and that causation has yet to be definitively demonstrated. However, this model could provide a useful test bed for unraveling pathophysiological mechanisms and evaluating new therapies.

GRANTS

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REFERENCES