The Effect of Vitamin E Succinate on Ischemia Reperfusion Injury

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Abstract Ischemia–reperfusion (I/R) injury is, in part, related to the burst of reactive oxygen species (ROS) generated after reperfusion. Vitamin E has been shown to exert its biological effects as an antioxidant, inhibiting the ROS. In this report, the effect of Vitamin E succinate (VES) on ischemia/reperfusion injury and NF- κ B expression was studied in a rat skeletal muscle model during reperfusion following a 4-h ischemic period. The study group consisted of muscle flaps infused with 150 mg/kg of VES given intraperitoneally 1 h post-initiation of ischemia. Muscle viability based on nitroblue tetrazolium staining, edema, and Doppler blood flow was measured in a control and a study group. Muscle samples were analyzed by standard gel shift assay. The VES experimental group showed an increase in muscle viability compared to controls (average of 44.675% versus 31.925%, respectively, $p=0.0415$). Blood flow, measured by Doppler 24 h after reperfusion, was increased in the VES study group compared to controls (10.3 vs. 5.1 ml/g/s $p=0.00355$). Additionally, the VES group showed a trend of decreasing edema compared with the control; however, not at a level that was statistically significant ($p=0.1267$). The VES-treated group showed a decreased expression of NF- κ B as compared to controls ($p<0.05$). These results show that vitamin E succinate has a protective effect in preventing I/R injury as measured by increased muscle viability and reperfusion blood flow. Vitamin E can exert its efforts as an antioxidant as well as other biological roles, including inhibition of NF- κ B.

Keywords Ischemia–reperfusion (I/R) injury · Reactive oxygen species (ROS) · Vitamin E succinate (VES) · NF- κ B · Laser Doppler nitroblue tetrazolium (NBT)

Introduction

Skeletal muscle injury often accompanies limb replantation and wound reconstruction with vascularized tissue transfers. The mechanism for this skeletal muscle damage is thought to be mediated by reperfusion of oxygenated blood to ischemic tissues [7]. The tissue ischemia results in cellular damage that is irreversible if prolonged. Ischemia to tissues results in cellular and ultrastructural changes that can lead to cell death. The restoration of blood flow has been associated with extension of the injury. Accumulating evidence indicates that cellular injury is caused by the burst of reactive oxygen species (ROS) generated after reperfusion [19]. Vitamin E has been shown to exert its biological effects as an antioxidant, inhibiting the ROS. The deprivation of oxygen results in the release ROS with resultant release inflammatory mediators as well as humoral mediators. The initial injury caused by the acute absence of oxygen is then further potentiated by progressive cellular death secondary to the inflammatory infiltration [17]. This occurs by release of inflammatory mediators that propagate the I/R injury through the induction of transcription factors [1, 16].

The transcription factor NF- κ B has been found to activate during the ischemic and subsequent reperfusion events [12]. NF- κ B is activated by the cellular cascade that occurs during reperfusion. The activation is initiated by cytokines and ROS-dependent mechanisms. Recent studies have shown VES to inhibit the transcriptional activity of NF- κ B [15]. In addition, VES has been found to block the

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binding activity of NF- κ B at the nuclear level. Vitamin E is able to exert its effects as an antioxidant as well as other biological roles, including inhibition of NF- κ B. I/R injury involves a variety of injurious events mediated by inflammatory cytokines and reactive oxygen species. In addition, multiple transcription factors are involved in the cascade of inflammation and cellular derangement. Any hope of counteracting these deleterious events will involve strategies aimed at interrupting the inflammatory cascade, in which NF- κ B appears to play a pivotal role. Interruption of this cascade would result in prevention of the cellular events that result in progressive cellular death.

Materials and Methods

Rat Gracilis Muscle Flap Model

Male Wistar rats (200–250 g, B&K Laboratories, Seattle, WA) were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneally) and with periodic supplementation (10 mg/kg intraperitoneally) as needed. A right thigh incision was made to expose the upper leg musculature and femoral vessels. The gracilis muscle was denervated and dissected free on its dominant vascular pedicles using standard microvascular technique (Fig. 1). Ischemia was induced by a microvascular clamp (O'Brien, Link America, Denville, NJ) carefully placed across the proximal femoral vessels. Muscle tissue utilized for biochemical analyses was harvested from the same identical area at the midsection of the gracilis muscle flap. Asepsis during the initial ischemia period was maintained by providing a local sterile environment. All experiments were approved by the Animal Care and Use Committee of Southern Illinois University and received humane care according to the "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, revised 1985). Two groups of animals were evaluated. Each group consisted of six animals ($n=6$). Group 1 was an ischemic control where animals underwent 4 h of ischemia followed by reperfusion. Group 2 animals

underwent 4 h of ischemia and treatment with intraperitoneal injections of VES (150 mg/kg) 1 h post-initiation of ischemia. The flaps were then perfused for 24 h, followed by flap harvest and animal euthanization. At the end of reperfusion, all animals were euthanized with concentrated pentobarbital (120 mg/kg i.v.)

Muscle Viability, Doppler Blood Flow, and Edema

Each group underwent 24 h of reperfusion prior to muscle flap harvest. Each sample then underwent evaluation for muscle viability, laser Doppler blood flow, and edema. To assess muscle viability, 3–4-mm sections were taken at the central portion of each muscle utilizing sharp dissection from the ischemic, as well as the contralateral muscles at the conclusion of the I/R challenge. Muscle viability was assessed on both cut surfaces using a previously described technique [3] employing nitroblue tetrazolium (NBT). Specimens were incubated in a solution containing 0.05% NBT and 0.2% TRIS buffer adjusted to pH 7.4 for 30 min at room temperature. The area of NBT staining was determined using digital planimetry (Sigma Scan Pro, Image Analysis Ver 5.0.0). The percentage of viability was then quantified by calculating the ratio of the total muscle surface area to the marked stained (viable) area. Both the ischemic flap and contralateral internal control gracilis muscles were assessed in order to ensure the uniform staining of muscles.

Laser Doppler blood flow was assessed after 24 h of reperfusion. A laser Doppler probe was placed on the vascular pedicle and flow was measured (ml/g/s). The measurements were obtained after a 1-min period of equilibration. The extent of muscle edema, a measurement of capillary membrane integrity, was assessed by tissue wet-to-dry weight ratios. Following 24 h of reperfusion, the animals were euthanized as above and the gracilis muscle flaps and contralateral non-ischemic controls harvested. The muscle flaps were weighed (wet weight) and then dried at 80°C in a convection oven for 36 h and reweighed (dry weight). Muscle edema was quantified by calculating the wet-to-dry weight ratio in each gracilis flap.

Figure 1 Gracilis muscle flap. The gracilis muscle was denervated and dissected free on its dominant vascular pedicles using standard microvascular technique. Ischemia was induced by a microvascular clamp (O'Brien, Link America, Denville, NJ) carefully placed across the proximal femoral vessels.

NF- κ B, Gel Shift Assay

Muscle samples were analyzed by standard gel shift assay. Cell extracts from muscle samples were incubated with a 32 P end-labeled DNA fragment containing the putative protein binding site for NF- κ B. The reaction products are then analyzed on a nondenaturing polyacrylamide gel. Gel band intensity was then analyzed by densitometry.

Results

The VES experimental group showed an increase in muscle viability compared to controls, average of 44.675% versus 31.925%, respectively ($p=0.0415$; Fig. 2). Blood flow, measured by Doppler 24 h after reperfusion, was increased in the VES study group compared to controls (10.3 vs. 5.1 ml/g/s, $p=0.00355$; Fig. 3).

In this study, a reduced amount of tissue edema (wet-to-dry weight of muscle) was seen consistent with the improved maintenance of membrane integrity. Sham-operated muscle showed a wet-to-dry weight ratio of $1.8 \pm \text{SEM } 0.32$. The VES-treated group showed a wet-to-dry weight ratio of $1.6 \pm \text{SEM } 0.11$. The VES group showed a trend of decreasing edema compared with the control; however, not at a level that was statistically significant ($p=0.1267$; Fig. 4).

Muscle samples were taken from experimental, and controls were analyzed via gel shift assay for NF- κ B levels. The VES-treated group showed a decreased expression of NF- κ B as compared to controls (Figs. 5, 6).

Figure 2 Graph of NBT staining. Muscle viability was assessed on both surfaces employing nitroblue tetrazolium (NBT). (Photograph of NBT staining). The percentage of viability as quantitated by calculating the ratio of the total muscle surface area to the marked stained (viable) area.

Figure 3 Graph of Doppler blood flow. Laser Doppler blood flow was assessed after 24 h of reperfusion. A laser Doppler probe was placed on the vascular pedicle and flow was measured (ml/g/s).

Discussion

This study showed that Vitamin E succinate was protective against I/R injury in a rat gracilis muscle model. Ischemia was induced via microvascular clamping of the pedicle to mimic those situations encountered in clinical practice, including complex wound reconstructions and limb replantation procedures. VES not only increased muscle viability as assessed by NBT staining but also resulted in increased blood flow to the pedicled muscle flap when compared to controls after. Importantly, the increase in blood flow was measured after 24 h of reperfusion and not just in the immediate reperfusion period. In addition, the VES study group muscle flaps had less edema compared to study groups. The decrease seen in muscle edema correlates with improvement in cell membrane integrity. Previous studies have shown capillary and post capillary venule dysfunction

Figure 4 Muscle edema. The muscle flaps were weighed (wet weight) and then dried at 80°C in a convection oven for 36 h and reweighed (dry weight). Muscle edema was quantified by calculating the wet-to-dry weight ratio in each gracilis flap.

Figure 5 NF-κB Electrophoretic gel. Gel band intensity. VES-treated samples (1A–3A) display decreased NF-κB expression compared to control samples (1B–3B).

in the microcirculation to be a major determinant in the manifestation and development of post-ischemic reperfusion injury [6, 14, 20]. Multiple mechanisms have been proposed to promote the progression of post-ischemic injury, including swelling of capillary endothelial cells, and increased extravascular pressure by interstitial edema [4, 8, 10, 11, 13]. This entire process is initiated by acute inflammation and the complex reactions that occur with reperfusion. Vitamin E, with its ability to neutralize reactive oxygen species, aids not only in the inhibition of the by-products of the ischemic event but also in the proinflammatory cascade that ensues upon reperfusion.

Vitamin E has been shown to function as an antioxidant with ability to inactivate reactive oxygen species. ROS are known to be important in the initial injury involved with I/R. The reperfusion event results in release of ROS, with resultant tissue damage. The infiltration of inflammatory cells and release of humoral mediators further potentiates the cellular damage [1]. In addition to necrotic death, apoptosis appears to be induced by the activation of signal transduction cascades promoted by the altered environment observed

during reperfusion [5]. The transduction cascades are a delayed process that can potentially be reversible if pathways can be interrupted. The initiation and propagation of I/R injury depends upon transcription factor activation which in turn is responsible for the induction of inflammatory genes required for the rapid production of proteins (cytokines, adhesion molecules, complement factors, and inducible nitric oxide synthase) [2, 16].

Vitamin E has been shown to function not only as an antioxidant but also as a regulator of signal transduction [15, 18]. NF-κB is thought to be the predominant transcription factor activated during reperfusion following an ischemic event [12]. Furthermore, reactive oxygen species are involved in the signaling cascade resulting in NF-κB activation [21]. NF-κB is implicated in the regulation of many genes involved in cellular adaptation mechanisms activated by I/R. NF-κB appears to be activated by cytokine and ROS-dependent mechanisms, operating only during reperfusion. The inhibition of NF-κB has been shown to protect against both oxidative stress in vitro [2] and I/R injury in vivo [9]. Recent studies have shown that VES has specific action in inhibiting the DNA binding activity of NF-κB in vitro [18]. VES was found to be the only compound to inhibit NF-κB DNA binding activity when compared to other related vitamin E structures [18]. NF-κB activation is induced via cell signaling pathways in I/R by TNFα with resultant ROS activation. The activated NF-κB then translocates to the nucleus of the cell, binding to a specific DNA sequence, with ensuing transcription. Mechanism of blockade of the DNA binding activity is postulated to be from VES's ability to oxidize various sulfhydryl groups, thus, disrupting binding activity. The ability of VES to interrupt this binding would prevent the downstream transcription and potentiation of the cellular damage and progressive cellular death. Further studies have shown VES to inhibit NF-κB transcription and translocation to the nuclei in vitro [15].

The results of this study confirm that VES has a role in the inhibition of NF-κB in our rat gracilis muscle model. This sheds light on the inflammatory cascade associated with U/R injury and the role NF-κB on its potentiation. Although Vitamin E has long been touted for its antioxidant properties, it now appears as if its effects are more far reaching. The results of this study show that vitamin E succinate is perfectly positioned in the biochemical pathways for the inhibition of ROS-dependent as well as ROS-independent mechanisms involved in I/R injury. The ability of VES to inactivate ROS, the ensuing inflammatory cascade, and the expression of harmful inflammatory gene products would provide a potential therapy in the prevention of skeletal muscle reperfusion injury. The prevention of muscle damage would result in improvement in muscle function. Hand function specifically could be improved by ensuring salvage of

Figure 6 NF-κB gel shift assay. The VES-treated group showed a decreased expression of NF-κB as compared to controls ($p < 0.05$).

function of the intrinsic musculature, as well as, the flexor or extensor balance in more proximal amputations or crush injuries requiring decompressive fasciotomy.

Conclusion

These results show that vitamin E succinate has a protective effect in preventing I/R injury as measured by increased muscle viability and reperfusion blood flow. Vitamin E can exert its effects as an antioxidant as well as other biological roles, including inhibition of NF- κ B. Improvement in muscle viability could result in better functional outcomes after ischemic challenge associated with crush injury or replantation.

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