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Ischemia Pre-conditioning and the Effects of Nitric Oxide Synthase

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THE ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE IN ISCHEMIC PRECONDITIONING OF A MUSCLE FLAP IN A RAT MODEL

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ABSTRACT

JESSICA JEAN HOLT: The Role of Inducible Nitric Oxide Synthase in Ischemic Preconditioning of a Muscle Flap in a Rat Model
(Under the direction of Dr. Kenneth Sufka)

Fifty male Sprague-Dawley rats were used in this study. The gracilis muscle flap with femoral vascular pedicle was dissected and used as a flap model. The ischemic preconditioning period consisted of three cycles of clamping the pedicle for 10 minutes followed by 10 minutes of reperfusion resulting in a total of 1 hour. In part I, the experimental group underwent the ischemic preconditioning for 1 hour. In the control group, the flaps were dissected without the clamping of the pedicle. Both groups were then subjected to 4 hours of global ischemia by continuous pedicle clamping, after which the flaps were sutured to their beds. Three days after the operation, the survival of the flaps was determined by gross and histological examinations. In part II, the experimental group underwent ischemic preconditioning, while the control group did not. The flaps from each group were harvested for iNOS gene expression using RT-PCR after 1 hour of perfusion and again after 4 hours of ischemia. The results indicated a significantly higher survival rate in the experimental group, with preconditioning than the non-preconditioned control group. iNOS gene expression was also significantly higher in the experimental groups than the control group at 1 hour after ischemic preconditioning. However, after 4 hours of global ischemia, iNOS expression in the control group was statistically higher than the experimental group. In conclusion, ischemic preconditioning can enhance flap tolerance to ischemic injury and improve flap survival rate. This study also provides evidence that the regulation of NOS may play an important role in the ischemic preconditioning phenomenon and thus the precise function of NOS in ischemia preconditioning warrants further investigation.

TABLE OF CONTENTS

Introduction

Microsurgical flaps have been widely used in clinical reconstructive surgery to successfully provide defect coverage while simultaneously restoring the form and function of the site of the defect without causing deformity of the donor site[1]. Though these procedures have achieved great clinical success, the incidence of flap failure still remains between 5 and 10 percent.[2,3] Prolonged ischemia and oxygen-derived free radicals induce peroxidation and cause ischemia-induced reperfusion injury during intra- or post-operative complications. They are thus believed to be the major cause of flap failure.[4,5] Ischemia is a low oxygen state usually due to obstruction of the arterial blood supply or inadequate blood flow leading to hypoxia and ultimately necrosis, or cell death, in the tissue. Ischemia occurs in microsurgical flaps because the pedicle, the source of artery and vein that represent the flap circulation to maintain tissue viability, must be ligated in order to transfer the flap from the donor site to the recipient site. During the time between the ligation of the pedicle and microsurgical anastomoses, or joining, of the pedicle to the recipient vessel, the flap experiences a lack of oxygen, or primary ischemia. After the flap has been transferred and the anastomosis of the pedicle to the recipient vessel is complete, thrombosis or clotting can occur at the site of anastomosis of the newly established vessel and cause secondary ischemia in the flap.[1] Increasing flap tolerance to ischemia-reperfusion injury may increase the possibility of salvaging the flap with complications.

While great efforts have been made to pharmacologically reduce ischemia reperfusion injury, research in the cardiology field found that brief periods of coronary artery occlusion, followed by episodes of reperfusion, could increase the resistance to further

ischemic damage in myocardial muscle.[6,7] This ischemic preconditioning technique has been applied to experimental and clinical microsurgical flap transfers. In animal muscle flap models, the survival of the muscle flap was improved if the main ischemic insult was preceded by a preconditioning session.[8,9] Preconditioning has also been found to be Geffective in decreasing muscle functional impair after prolonged ischemia.[10,11]

The mechanism of the effect of ischemic preconditioning phenomenon on flap survival is still under intense investigation. One theory involves the ability of ischemic preconditioning to increase functional capillary perfusion and reduce leukocyte-mediated inflammation in the tissue with reperfusion injury.[12-14] Another theory is based on the molecular mechanisms involved in ischemia preconditioning injury such as the phosphorylation of the adenosine triphosphate-dependent potassium channel (K_{ATP} channel) which increases the K+ efflux and thus exerts their cardioprotective effect. Pre-conditioning is an adaptation triggered by many different chemical signals during the original ischemic stress. The chemical messengers can include Adenosine, bradykinin, catecholamines, and nitric oxide, all of which have been reported to exert their effects on these K_{ATP} channels.[13,15] Because so many chemicals trigger preconditioning, the removal of one, such as Nitric Oxide, does not block the development of protection in the presence of other stimuli. However, exogenous supplementation of Nitric Oxide, such as SNAP or nitroglycerin, can produce a pre-conditioning response against ischemia.[15] According to the nitric oxide hypothesis of late preconditioning, ischemic preconditioning occurs in two phases, termed the early and late phases of preconditioning. The early phase of preconditioning occurs immediately after the initial ischemic insult and lasts 2-3 hours, while the late phase of preconditioning begins 12-24

hours later and lasts three to four days.[16] Both phases of preconditioning are useful in a clinical setting. The early phase of preconditioning would provide protection against primary ischemia, while the late phase would be associated with the protection against secondary ischemia. The two-phase process of preconditioning is due to the mechanism by which nitric oxide provides ischemic protection. Nitric Oxide plays a prominent role in both initiating an adaptive response when a preconditioning stimulus is applied and mediating the protection hours later.[15,16] According to this hypothesis, a brief episode of ischemic stress causes increased production of Nitric Oxide (NO) and reactive O_2- probably by eNOS. NO and the reactive oxygen species activate protein Kinase C epsilon (PKCε) via an unknown mechanism. PKCε then triggers a complex signaling cascade involving Src and Lck tyrosine kinases, leading to the phosphorylation of $I_kB\alpha$ and mobilization of the transcription factor NF-$_K$B. Other transcription factors are thought to be involved including the JAK/STAT signaling pathway. STAT has been determined to be required for iNOS regulation. Binding of NF-$_K$B and STAT to the iNOS promoter results in the activation of the promoter and the transcription of the iNOS gene, thus increasing synthesis of the iNOS protein. An increased production of the iNOS protein results in enhanced NO biosynthesis and a preconditioned phenotype.[15,16] Other studies have agreed that NO is responsible for the protective preconditioned state, but have also concluded that the accumulation of NO is harmful. They suggest that NO preconditioning (or iNOS inhibitors) blockades the overproduction and thus accumulation of NO that is harmful to the tissue.[17] While these important studies focused on myocardium, little research has been reported to apply these studies to skeletal muscle pre-conditioning. In this study we examined the molecular marker of NO to determine its

role in correlation with ischemic preconditioning on improving muscle flap survival in a rat model.

I. Materials and Methods

Fifty male Sprague-Dawley rats weighing between 380-420 grams and cared for under the National Research Council's guidelines for the care and use of laboratory animals were used in this study. The rats were anesthetized using pentobarbital administered by intraperitoneal injection (50 mg/kg). Following induction of general anesthesia, the lower abdomen and groin were shaved, and the animals were placed in a supine position.

Flap model and preconditioning protocol

The right groin vessels and gracilis muscles were exposed by using a transverse groin incision. The gracilis muscle flap was elevated with femoral vascular pedicle.[18] The preconditioning protocol was designed based previous protocol investigating preconditioning in the rat flap models.[19] The treatment muscle flap underwent ischemic preconditioning by 10 minutes of pedicle clamping using Acland V3 clamps followed by 10 minutes of reperfusion. This ischemia/reperfusion sequence was repeated three times for a total preconditioning period of 1 hour. The experiment was divided into two parts.

Part I. Flap survival

Twenty rats were divided into two groups. In the experimental group (n=10), the muscle flap underwent the ischemic preconditioning procedures for 1 hour. Then the muscle flap was subjected to 4 hours of global ischemia induced by pedicle clamping. In the control

group, the muscle flap was simply perfused without clamping for 1 hour after dissection, and then subjected to 4 hours of ischemia. After ischemia time, the clamps were released, and the flaps from both groups were fixed to their original location, after which the overlying skin was sutured with running 4-0 Nylon sutures.

The animals were allowed to recover in their individual cages a period of 72 hours. After this time, the animals were once again anesthetized as described above. Survival of the flaps was determined by gross examination of their color, texture, and vascular supply. The flaps were then removed and placed in 10% formalin for histology.

Part II. Inducible nitric oxide synthase (iNOS) gene expression

Thirty rats were used in this part of the study. The animals were divided into two groups as above. In the experimental group (n=12), the muscle flaps underwent preconditioning procedures followed by an ischemia period. Six flaps were harvested at the end of 1 hour of preconditioning and others were harvested at the end of 4 hours of global ischemia for examination. In the control (n=12), six flaps were harvested at the end of 1 hour perfusion and the remaining six were harvested at the end of 4 hours global ischemia. The samples from normal gracilis muscle (n=6) were examined for the baseline of gene expressions.

RT-PCR

iNOS mRNA from the muscle samples of each group were examined. The biopsied tissues were homogenized, and the total RNA was isolated from the skin using Trizol reagent (GIBCO BRL, Gaithersburg, MD). The extracted RNA was then reverse

transcribed into cDNA. The reaction was performed in a final volume of 25 μL, consisting of 2.5 μL 10× PCR buffer, 1.5 μL of 25 mM $MgCl_2$, 2μl mixture of 1 mM dNTP, 0.125 μl of Taq DNA polymerase (5U/μl), 1.5 μL of each primer 50 μM, and 1 μL of cDNA. The typical PCR reaction conditions were 2 minutes at 94 °C, and then 35 cycles at 94 °C for 50 seconds, 60 °C for 50 seconds, and 72 °C for 50 seconds. After the cycles were complete, the reactions continued with conditions at 72 °C for 10 minutes and then remaining at 4 °C . The primer was obtained commercially (The Midland Certified Reagent Company, Midland, TX). PCR products were subjected to 10% polyacrylamide gel electrophoresis. The gel with the separated DNA bands were photographed and scanned. To ensure that equal amounts of reverse-transcribed cDNA were applied to the PCR reaction, the primer pairs for β-actin, a constitutively expressed gene, were also included in the PCR reaction. Quantitative gene expression was expressed as fraction of iNOS to β-actin.

After flap survival examination and tissue biopsies, the animals were sacrificed by an overdose of pentobarbital. The data on flap survival from different groups were compared using a chi-square test. The data on gene expression from different groups were compared using one-way analysis of variance (ANOVA) for multiple testing. Statistical significance was assumed at $p<0.05$.

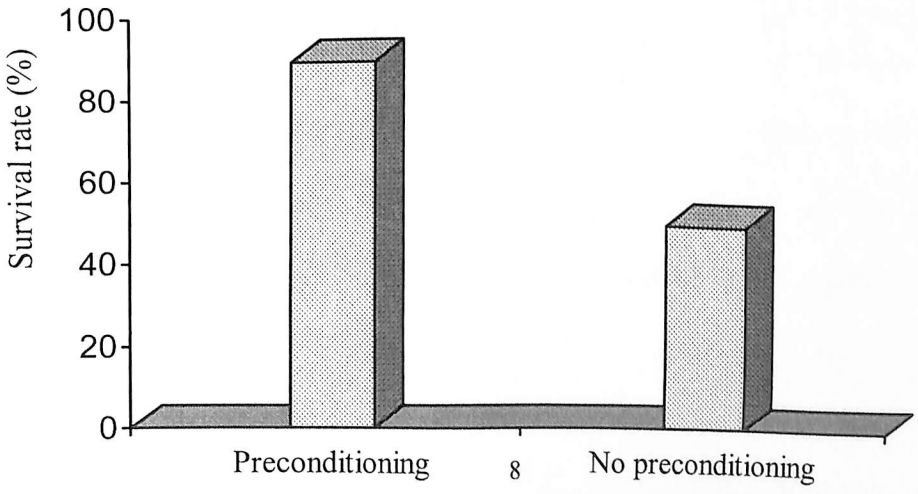
II. Results

Flap survival

The surviving flaps were pink, pliable and appeared well perfused. The consistency of the muscle was normal and it displayed healthy circulation as evidenced by the patent vascular pedicle and by cutting into it with a scalpel. In contrast, non-viable flaps were distinguished based on the lack of circulation in the flap and the extensive thrombosis in the vascular pedicle. These flaps were pale in appearance, as well as being very thin and firm relative to the surviving ones.

The results of flap survival are shown in Figure 1. After 4 hours ischemia, 9 of the 10 flaps in the experimental group that had undergone ischemic preconditioning survived, while only 5 of the 10 flaps survived in the control group without preconditioning. There was a significant difference between the survival rates of these two groups ($p<0.05$).

Figure 1 - Comparison of flap survival



Histology

Histological analysis confirmed that the flaps that were determined to have survived based on gross examination were in fact alive and viable. Among the surviving flaps, the muscle fibers were more organized with less evidence of damaged myocytes and architectural disarray. The capillaries appeared normal and showed no indication of endothelial damage. The muscle of non-viable flaps had loss of striations, fragmentations and nuclear dropout indicating that extensive myonecrosis had occurred.

Gene expression

Gene expression was expressed as a fraction of growth factors to β-actin (Mean ± standard deviation). The expression of iNOS was detected in the flaps. The iNOS expression from normal gracilis muscle was 0.27 ± 0.09. In the experimental group, the mean iNOS expression from the flaps was 0.73 ± 0.18 after 1 hour ischemic preconditioning, which was significantly higher than iNOS from the control flaps with 1 hour perfusion without preconditioning (0.26 ± 0.11) and the normal muscle samples ($p<0.01$). However, after 4 hours global ischemia, the fraction of iNOS expression in the flaps with preconditioning was significantly decreased to 0.26 ± 0.07 while iNOS in the control was increased to 0.83 ± 0.16. The changes of iNOS expressions of these two groups are shown in Figure 2 and 3.

Figure 2 - iNOS gene expression in flaps with ischemic preconditioning

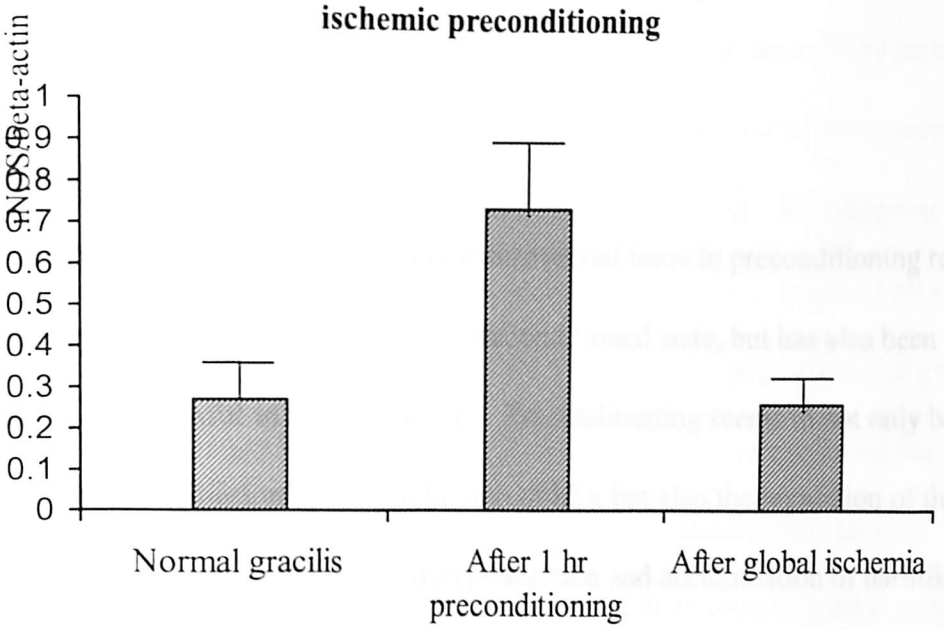
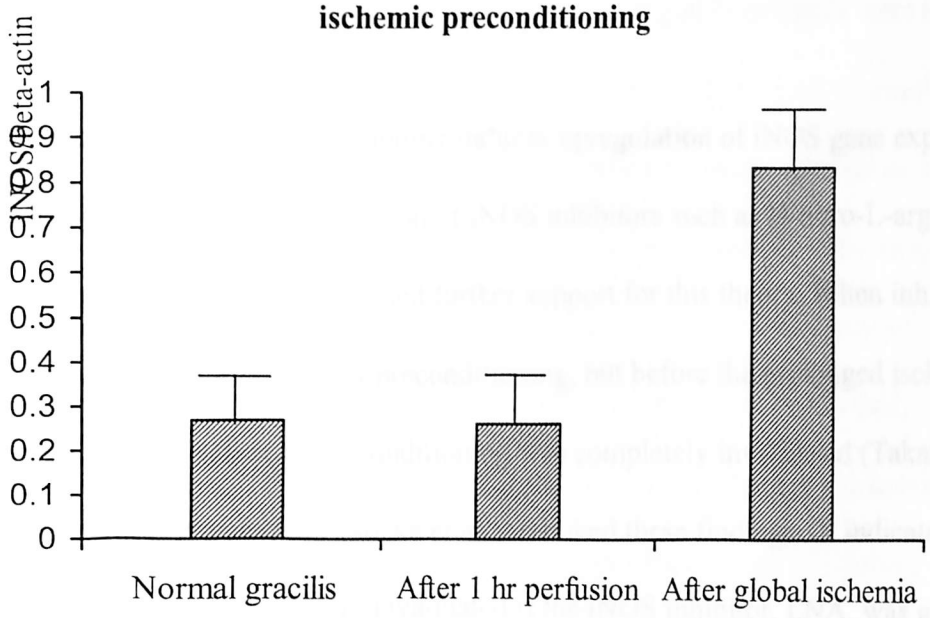


Figure 3 - iNOS gene expression in flaps without ischemic preconditioning



III. Discussion

Nitric Oxide has been a very popular and controversial issue in preconditioning research. It has been found to initiate the protective preconditioned state, but has also been determined to be harmful in accumulation.[17] Preconditioning seems to not only be responsible for the initiation of the production of NO, but also the regulation of the production and thus prevention of the overproduction and accumulation of harmful levels of NO although the precise mechanism is still under investigation.[17] The detrimental role of NO in accumulation seems to be due to the formation of highly reactive oxidant species.[20]

It has been proposed that preconditioning induces upregulation of iNOS gene expression and thus NO sythesis. The introduction of iNOS inhibitors such as N-nitro-L-arginine (LNA) or aminoguanidine has provided further support for this theory. When inhibitors such as these were introduced after preconditioning, but before the prolonged ischemic period, the protective effect of preconditioning was completely invalidated (Takano and [15,21]). However, the research of Csonka et al.[17] rebuked these findings. It indicated that the effects of preconditioning were invalidated if the iNOS inhibitor, LNA, was given prior to preconditioning, but that the protective preconditioned state was unaltered if LNA was given after preconditioning but before the prolonged ischemic period.[17] Therefore it was determined that both preconditioning or an appropriate concentration of iNOS inhibitor decreases injury due to ischemia as is indicated by an enhanced post-ischemic function. [17]

The role of nitric oxide in preconditioning as stated by the nitric oxide hypothesis of late preconditioning was further supported by this study.[16,17] The amount of NO present after 1 hour of preconditioning was significantly higher than the amount of NO present in the control non-preconditioned muscle flaps with 1 hour of perfusion. This supports the hypothesis that the protective effect of ischemia preconditioning is triggered by an increase in NO.[16,17] The decreased levels of NO accumulation after 4 hours of global ischemia in preconditioned flaps versus the NO levels in non-preconditioned flaps again supports previous research when the survival of these flaps is taken into account.[13,17] The preconditioned flaps that had lower levels of iNOS after four hours of global ischemia were found to be alive and viable, while the non-preconditioned flaps that had significantly higher levels of iNOS after the four hours of global ischemia were nonviable and had extensive myonecrosis. Thus one might conclude that ischemia preconditioning induces an iNOS cascade that in turn preconditions the tissue against further ischemia by preventing overproduction of NO during subsequent ischemic periods. This mechanism seems to occur much in the same way that vaccines prevent the development of illness by exposing the body to small amounts of harmful substance in order that the body might develop its own defense. However, the precise mechanism by which the ischemia preconditioning induced iNOS prevents the harmful accumulation of NO during subsequent ischemic periods is yet to be determined and thus would warrant further investigation.

IV. Conclusion

Ischemia preconditioning is useful in preventing injury and loss of function due to ischemic periods in muscle flaps, and Nitric Oxide Synthase has been determined to have a vital role in the mechanism by which preconditioning prevents this ischemic injury. However, the specific pathway by which iNOS functions is not yet fully understood. Perhaps through further investigation of preconditioning and the function of iNOS in the preconditioning event, injury due to ischemia can be prevented clinically through the use of iNOS inducers and inhibitors and the careful monitoring of iNOS levels within the flap tissue.

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