Research Article

The Effect of Ischemia Reperfusion on Intestinal Contractility Regulated by the Nitrergic System

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Abstract: Because of the interaction between nitric oxide (NO) and the superoxide anion radical, the effect of ischemia-reperfusion on intestinal contractility regulated by the nitrergic system was investigated in the present study.

The study was performed on 3 groups of rabbits: group 1, ischemia; group 2, ischemia and 1 h of reperfusion; group 3, ischemia and 24 h of reperfusion. Tissue samples were obtained from ischemic, ischemia-reperfused and adjacent uninjured intestines as study and control samples. The effects of atropine, tetrodotoxin, L-NAME and L-arginine on the intestinal response to electrical field stimulation (EFS) were investigated. Guanethidine was used to minimize adrenergic activity.

Tetrodotoxin and atropine prevented contractions. L-NAME enhanced the responses to EFS in all tissue samples except for the study tissue of group 2, and L-arginine reversed this contraction elevation. Group 2 study tissue response was as high as 170% of that of the control tissue in standard Krebs-Henseleit solution, and no change was seen on this level with L-NAME and L-arginine addition.

The effects of tetrodotoxin and atropine revealed that EFS affects via the cholinergic neuronal system. Ischemia reperfusion affects intestinal contractility, especially in the early phases of reperfusion. In the light of the increased response to EFS and insensitivity to L-NAME and L-arginine of the affected tissue during this period it was thought that the nitrergic system is considerably affected by ischemia reperfusion. Excessive production of superoxide anion radicals or reversible inhibition of nitric oxide synthase may be the cause of this.

Key Words: Intestinal contractility, intestinal ischemia reperfusion, L-arginine, L-NAME, nitric oxide

İskemi Reperfüzyonun Nitrerjik Sistem Tarafından Düzenlenen Bağırsak Kontraktilitesine Etkisi

Özet: Bu çalışmada, nitrik oksit (NO) ve süperoksit anyon radikali arasındaki etkileşim nedeniyle nitrerjik sistem tarafından düzenlenen bağırsak kontraktilitesine iskemi-reperfüzyonun etkisi incelendi.

Çalışma, 1. grup iskemi; 2. grup iskemi ve 1 saat reperfüzyon; 3. grup iskemi ve 24 saat reperfüzyon şeklinde dizayn edilen 3 grup tavşan üzerinde gerçekleştirildi. İskemi ve iskemi-reperfüzyon dokularından çalışma, bunların komşuluğundaki sağlıklı bölgeden kontrol doku örnekleri alındı. Bağırsağın elektrik alan stimulasyonuna (EAS) cevabı üzerine atropin, tetrodotoxin, L-NAME ve L-arginin'in etkileri incelendi. Adrenerjik etkiyi önlemek için guanetidin kullanıldı.

Tetrodotoxin ve atropin, kontraksiyonları önledi. L-NAME, ikinci grup çalışma dokusu hariç tüm dokularda EAS'na cevapları artırırken, L-arginin bu kontraksiyon artışlarını önledi. İkinci grup çalışma dokusu cevabı "Krebs-Henseleit" içindeki kontrol dokusunun % 170'i kadardı ve bu düzeyde, L-NAME ve L-arginin ilavesiyle bir değişiklik gözlenmedi.

Tetrodotoxin ve atropin'in etkileri, EAS'nun etkisinin kolinerjik sinir sistemi aracılığıyla olduğunu gösterdi. İskemi-reperfüzyon, intestinal kontraktiliteyi özellikle reperfüzyonun erken döneminde etkilemektedir. Bu dönemde etkilenmiş dokudaki EAS'na cevap artışı ve L-NAME ile L-arginine cevap oluşmaması, nitrerjik sistemin iskemi-reperfüzyondan önemli ölçüde etkilendiğini düşündürmektedir. Bunun sebebi aşırı süperoksit anyon radikali üretimi veya nitrik oksit sentaz enziminin geri dönüşlü inhibisyonu olabilir.

Anahtar Sözcükler: Intestinal kontraktilite, intestinal iskemi reperfüzyon, L-arginin, L-NAME, nitrik oksit

Introduction

Intestinal ischemia leads to consumption of the energy sources of the cell by blocking energy production. Energy insufficiency is the etiology of the subsequent pathologic events. Consumption of ATP leads to accumulation of purine bases such as hypoxanthine and xanthine. The resultant decrease in energy leads to ion pump insufficiency, which causes Ca^{+2} ion accumulation that plays a role in xanthine dehydrogenase conversion to xanthine oxidase. These events trigger reperfusion injury upon reoxygenation. When oxygen enters ischemic tissue, hypoxanthine-xanthine-uric acid turnover is catalyzed by xanthine–oxidase. H_2O_2, which is a product of this reaction, is the pioneer of other oxygen free radicals. These free radicals attack all biomolecules. Deterioration at the molecular level leads to structural and functional alterations in tissue and organ systems, constituting reperfusion injury. Reperfusion injury significantly affects the intestinal mucosa and villi (1). This injury also leads to alterations in the motor function of the intestine (2-4).

Irregularity of peristaltic waves and lengthening of intestinal transit time are observed after ischemia-reperfusion (I/R) (4). Intestinal motility is under the control of neural and humoral factors. The cholinergic system increases motility. One important non-adrenergic-non-cholinergic (NA-NC) inhibitor system is the nitrergic neurons (5). Nitric oxide (NO) is produced by nitrergic neurons, leukocytes, the epithelium, smooth muscle cells and intraluminal bacteria from arginine, by nitric oxide synthase (NOS) (5). NO is an inorganic, lipophylic, unstable free radical that can diffuse easily through the membrane without dependence on a receptor. NO and the superoxide anion radical (O_2^{-}), which is produced abundantly during reperfusion, react and transform to peroxinitrites. This reaction has a scavenging effect on O_2^{-}, although peroxinitrites are also hazardous molecules. On the other hand, this reaction consumes NO in tissue.

Because of the reaction between NO and O_2^{-} the effect of I/R on intestinal contractility regulated by the nitrergic system was investigated in the present study, as measured by the contractile response to electrical field stimulation (EFS) of the intestine.

Materials and Methods

Twenty-seven New Zealand white rabbits (weighing 1500 to 3000 g) were used in the study. The study was performed on 3 groups of animals, with 9 animals in each group: group 1, ischemia; group 2, ischemia and 1 h of reperfusion; and group 3, ischemia and 24 h of reperfusion. Rabbits were kept under standardized conditions for food, water, light, and temperature before and during the study.

An ischemia reperfusion model that had been used before successfully was applied (1). Overnight fasted animals were anesthetized with ketamine hydrochloride (25 mg/kg) and anesthesia was maintained through intravenous infusion of sodium pentobarbital (25 mg/kg) via the ear vein during the study. A midline laparotomy was performed after preparation of the abdominal wall with 10% povidone iodine solution. The mesentery artery that perfused a 30 cm segment of the distal ileum was exposed. An atraumatic microvascular clamp was placed across the artery, avoiding occlusion of the studied segment's vein. The marginal vessels at both ends of the segment were divided and ligated, and the intramural collateral blood flow was stopped with atraumatic intestinal clamps. Mesenteric ischemia was confirmed when the mesenteric pulsations were lost and the intestinal segment became pale. The bowel was returned to the abdominal cavity, and the incision was closed with interrupted 2/0 silk sutures. After 60 mins of ischemia the study was terminated in group 1. In the other groups, reperfusion was performed by removing all of the clamps. Mesenteric reperfusion was confirmed by the return of pulsation and color. The bowel was then returned to the abdominal cavity once more, and the incision was closed with interrupted 2/0 silk sutures. After 1 h of reperfusion in group 2 and 24 h of reperfusion in group 3, the experimental procedure was terminated and the animals were sacrificed. The animals in group 3 were awakened after reperfusion was performed, and reanesthetized after 24 h. Tissue samples were obtained from ischemia-reperfused and proximally adjacent intact intestines as study and control samples, respectively.

Segments (1 cm in length) from experimental and normal ileum were cut and the lumen was washed with Krebs-Henseleit solution to remove any traces of food. Segments were mounted between a pair of parallel platinum electrodes and placed in 25 ml organ baths containing Krebs-Henseleit solution, aerated with 95% O_2 and 5% CO_2 and maintained at 37 $^{\circ}$C. The Krebs-Henseleit solution was composed of (mM) NaCl 119, KCl

4.7, $MgSO_4$ 1.5, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11. The testing tension was adjusted to 2 g, and the tissues were allowed to equilibrate for 1 h before the start of experiments. During the equilibration period, the bathing medium was replaced every 15 min. The Krebs-Henseleit solution always contained guanethidine (10^{-5} M) to minimize adrenergic activity in response to EFS. The tissues were subjected to EFS via parallel platinum electrodes by a dual impedance stimulator (Harvard) to obtain nerve-mediated contractions of ileal segments. The contractions were recorded isometrically using an oscillograph (Harvard).

After the equilibration period, ileal segments were constricted with KCl (80 mM). The voltage required to elicit a similar amplitude of the maximum KCl contraction was determined in control tissues, and this voltage was subsequently used in both control and study tissues in all groups.

After control responses to EFS (0.3 ms, 10 Hz) the ileal segments were incubated with a single concentration of atropine (10^{-5} M, 10 min), L-arginine (10^{-3} M, 20 min), L-NAME (10^{-4} M, 20 min), or tetrodotoxin (TTX, 10^{-6} M, 10 min) and EFS was repeated at 2 min intervals after at least 3 consistent contractions had been obtained. We observed separately the effects of atropine, TTX, L-NAME and L-NAME + L-arginine on the response to EFS using different preparations. Responses were calculated as a percentage of the responses to the KCl obtained at the beginning of the study.

Statistical Analysis: The results were evaluated by SPSS for Windows 6.0. The differences between groups were analyzed by the Kruskal-Wallis test, and the Mann Whitney U test was used to show whether those differences were present and between which groups. Statistical significance was defined as $P < 0.05$.

Results

The adrenergic system had no effect on the results during the experimental periods since guanetidine was present in the medium. Contractions resulting from EFS were totally prevented by TTX, demonstrating that these contractions were the result of neural stimulus rather than direct muscular stimulus. Atropine prevented about 91% of contractions in all animals, supporting the role of the cholinergic system in this process.

There was no difference between the control and study tissue responses to EFS in the standard organ bath in the ischemia or the 24 h reperfusion groups. Study tissue response was as high as 170% of that of the control ($P < 0.05$) in the 1 h reperfusion group (Table 1).

A significant increase was observed in the first and third group EFS responses when L-NAME (a competitive inhibitor of nitric oxide synthase) was added to the medium ($P < 0.05$) (Figure 1). In the second group, EFS response was high but there was no difference in experimental tissue responses before and after L-NAME ($P > 0.05$) (Figure 1, Table 2).

When L-arginine (NOS substrate) was added to the medium containing L-NAME, the increased contraction responses of all the tissues decreased to the level of the starting point except for in the 1 h reperfusion group (Figure, Table 3).
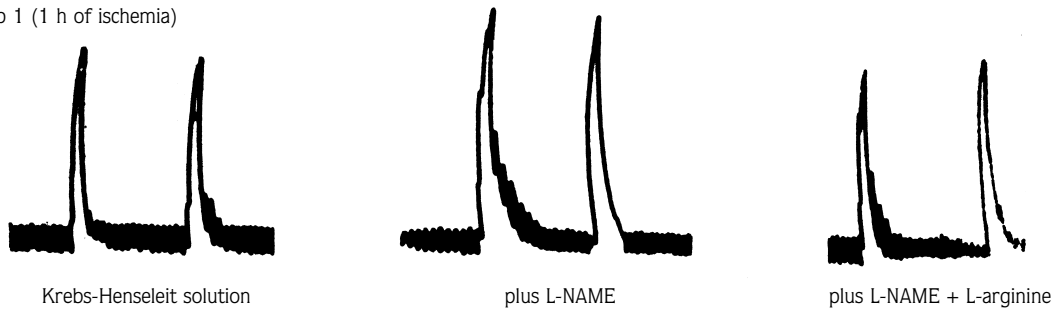
EFS responses of control and study tissues of the ischemia and 24 h reperfusion group were very similar, but that of the study tissue of the 1 h reperfusion group was not sensitive to L-NAME or L-arginine.

Table 1. EFS results of control and study tissues in standard Krebs Henseleit solution (as percent of the basic contraction level; mean ± SD).

n: 9	Group 1	Group 2	Group 3
Control tissue	98.8 ± 5.85	99.3 ± 4.83	98.8 ± 4.25
Study tissue	103 ± 7.53	169.2 ± 8.23*	105.7 ± 9.42

* Significantly different from the control and the other 2 groups (P < 0.05)

Table 2. EFS results of control and study tissues in standard Krebs Henseleit solution plus L-NAME (as percent of the basic contraction level; mean ± SD).
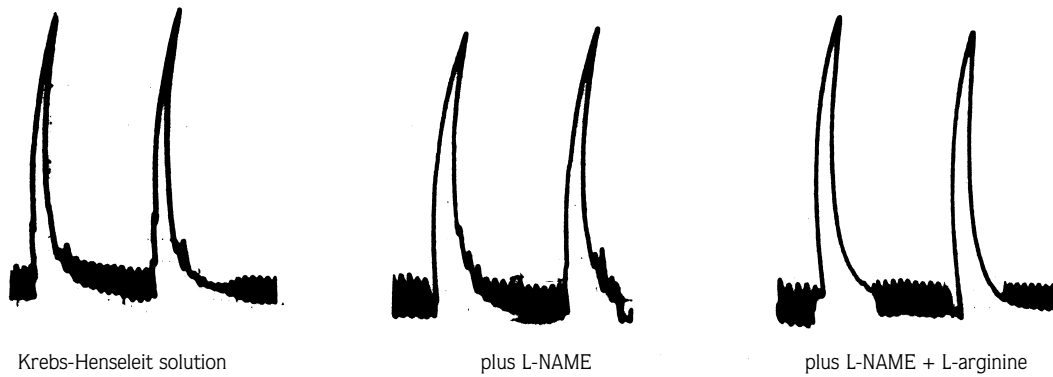
n: 9	Group 1	Group 2	Group 3
Control tissue	131.5 ± 6.53	136.5 ± 7.50	134.8 ± 8.89
Study tissue	129.5 ± 3.27	173.5 ± 5.43*	141.0 ± 4.73

* Significantly different from the control and the other 2 groups (P < 0.05)

1a: Group 1 (1 h of ischemia)

| Krebs-Henseleit solution | plus L-NAME | plus L-NAME + L-arginine |

1b: Group 2 (1 h of reperfusion)

| Krebs-Henseleit solution | plus L-NAME | plus L-NAME + L-arginine |

1c: Group 3 (24 h of reperfusion)

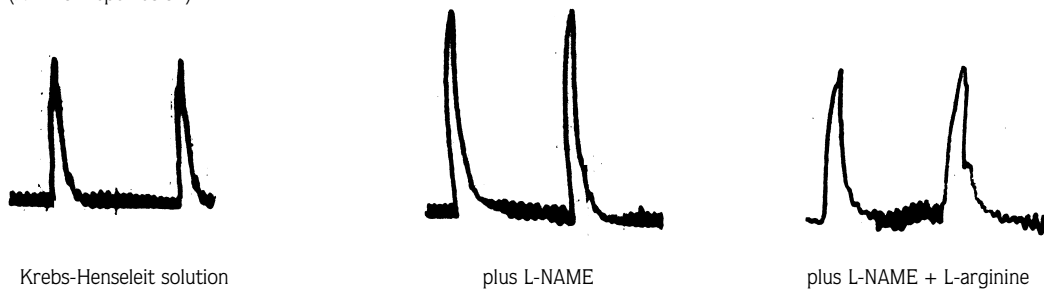| Krebs-Henseleit solution | plus L-NAME | plus L-NAME + L-arginine |

Figure. Oscillograph records of the study tissue samples.

Table 3. EFS results of control and study tissues in standard Krebs Henseleit solution plus L-NAME + L-arginine (as percent of the basic contraction level; mean ± SD).

n: 9	Group 1	Group 2	Group 3
Control tissue	106.7 ± 6.83	102.2 ± 6.34	103.0 ± 7.75
Study tissue	107.8 ± 5.49	170.5 ± 6.02*	110.0 ± 7.07

* Significantly different from the control and the other 2 groups (P < 0.05)

Discussion

Oxygen free radicals, which are primarily responsible for I/R injury, are also responsible for the motility disorders of the gastrointestinal system after I/R (2). It is reported that antioxidants prevent these disorders (2,3) and that inhibition of the antioxidant system increases them (2). It is thought that oxidative stress destroys the regulation of intestinal motility.

Injury of the gastrointestinal system is directly related to the duration of I/R. Integrity of intestinal motility

shows a negative correlation with the duration of ischemia but a positive correlation with reperfusion time (4).

Disarrangement and an increase in intestinal contractions have been demonstrated in the early phases of reperfusion after an ischemic event (3,6,7). Moody et al. (8) reported motor coordination disarrangement at 2 h after a hypovolemic shock at a duration of 60 min and partial recovery after 24 h. Alican et al. (6) reported a delay in intestinal transit time starting at the 2nd hour of reperfusion and lasting 24 h after 30 min of ischemia.

Intestinal peristalsis is controlled by the neural and hormonal systems. The neural system consists of cholinergic, adrenergic and nonadrenergic-noncholinergic systems. NO is an important mediator in the NA-NC system. NO related reflex proximal contractions and distal relaxations produce the peristaltic waves that are mandatory for the transport of intestinal contents. Intestinal transit time increases when the nitrergic system is affected. EFS stimulates both the cholinergic and nitrergic neurons (9). NO reduces the secretion of acetylcholine from cholinergic neurons and causes relaxation by affecting the muscle directly (9,10). Decreased NO cancels the inhibition system, leading to increased contractions. Enzyme studies show the effect of NO on motility in intestinal tissues. L-NAME is a competitive inhibitor of NOS and leads to an increase in tonic and clonic contractions in the colon (11). This effect of L-NAME can be reversed with the addition of the NOS substrate L-arginine (11). NO produces nonradical compounds with superoxide. Superoxide dismutase (SOD) increases the half–life of NO (12-14). Dysfunction of NO is important in I/R where free radicals and lipid peroxidation are produced. Oxygen free radicals, which are early products of I/R, consume NO and impair the intestinal smooth muscle contractions that are arranged by NO.

Ischemia with a duration of 1 h had no significant effect on EFS response but a significant increase in contractions was seen in the 1 h reperfusion group. The study tissue contraction level was equal to that of the control tissue at 24 h of reperfusion. This indicates that injury after 1 h of reperfusion is reversible after 24 h.

L-NAME addition increased control and study tissue EFS responses in all groups except for the 1 h reperfusion group study tissue. Contraction levels decreased to baseline levels after the addition of L-arginine. These results show that the nitrergic system has a regulator effect on intestinal motility and that L-NAME increases the susceptibility of intestinal muscle tissue to the cholinergic stimulus. On the other hand, 1 h reperfusion tissue revealed the highest response to EFS, and neither L-NAME nor L-arginine had any effect on this response. NOS inhibition due to I/R injury may be the cause of this, because these tissues showed an increased sensitivity to EFS and L-arginine was unable to induce any NO dependent decrease on the contraction level. This change was temporary, because the 24 h reperfusion tissue responses in the presence of L-NAME and L-arginine were the same as those in the control tissues.

I/R affects intestinal contractility, especially in the early phases of reperfusion. This effect is similar to that of the inhibition of NO synthesis in the healthy intestine, but is reversed within 24 h. We think that reversible NOS inhibition is the main cause of this. Another possible reason may be the consumption of NO by oxygen free radicals, but this can not explain the inefficacy of L-arginine.

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