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Ischemic Preconditioning and Tacrolimus Pretreatment as Strategies to Attenuate Intestinal Ischemia-Reperfusion Injury in Mice

P. Stringa, D. Romanin, N. Lausada, M. Machuca, J.C. Raimondi, A. Cabanne, M. Rumbo, and G. Gondolesi

ABSTRACT

The intestine is highly sensitive to ischemia-reperfusion injury (IRI), a phenomenon occurring in different intestinal diseases. Several strategies to mitigate IRI are in experimental stages; unfortunately, no consensus has been reached about the most appropriate one. We report a protocol to study ischemic preconditioning (IPC) evaluation in mice and to combine IPC and tacrolimus (TAC) pretreatment in a warm ischemia model. Mice were divided into treated (IPC, TAC, and IPC + TAC) and untreated groups before intestinal ischemia. IPC, TAC, and IPC + TAC groups were able to decrease postreperfusion nitrites levels ($P < .05$). IPC-containing groups had a major beneficial effect by preserving the integrity of the intestinal histology ($P < .05$) and improving animal survival ($P < .002$) compared with TAC alone or the untreated group. The IPC + TAC group was the only one that showed significant improvement in lung histological analysis ($P < .05$). The TAC and IPC + TAC groups down-regulated intestinal expression of interleukin (II)-6 and IL1b more than 10-fold compared with the control group. Although IPC and TAC alone reduced intestinal IRI, the used of a combined therapy produced the most significant results in all the local and distant evaluated parameters.

ISCHEMIA-reperfusion injury (IRI) of the intestine is part of the pathophysiology of many intestinal disorders, such as strangulated hernia, volvulus, necrotizing enterocolitis, mesenteric embolic event, procoagulant disorders, and intestinal transplantation. It is an important factor associated with morbidity and mortality in both surgical and trauma patients.[1] IRI is a dynamic process involving two distinctive yet interrelated phases of ischemic organ damage and inflammation-mediated reperfusion injury. Multiple cellular and molecular pathways contribute and regulate tissue/organ damage, eg, the exposure of vascular neoantigens interacting with complement-activating natural antibodies, and the uncontrolled generation of reactive oxygen species and proinflammatory mediators.[1–3] A hallmark of intestinal IRI is epithelial cell damage, accompanied by loss of brush border enzymes and absorptive function. In the case of the intestine, IRI may alter the integrity of the mucosal enteric barrier, promoting bacterial translocation and sepsis. Pro-inflammatory factors, such interleukin-1 beta (IL-1b), interleukin-18 (IL-18), and other cytokines, are produced in the intestine during IRI, contributing to a local and systemic inflammatory response leading to damage in remote organs, such as the liver and lungs, causing multiorgan failure and death.[4] Strategies to mitigate IRI must be designed for basic and translational research in the intestinal surgical field including transplantation. Several strategies have been proposed to protect tissues from IRI, such as antioxidant administration, hypothermia, inflammatory mediator or adhesion molecule modulation, ischemic preconditioning (IPC), or different drug therapies.[5] In 1986,

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Murry et al[6] introduced the IPC concept as a way to reduce myocardial IRI. They described the beneficial effect of short periods of coronary occlusion followed by short periods of reperfusion, before a prolonged ischemic phase, and found a reduction of ischemic myocardial injury in dogs. IPC has been studied in different tissues and organs, including the intestine, since it was first described.[7]

Tacrolimus (TAC) is a macrolide antibiotic compound, a metabolite of the fungus *Streptomyces tsukubaensis*, discovered in 1984. Potential applications of this drug are still under investigation. Favorable results have been obtained with the use of TAC in various immune-mediated phenomena, including inflammatory bowel disease and solid organ rejection prevention and treatment.[8] Several studies have shown that TAC can ameliorate IRI by slowing adenosine triphosphate (ATP) depletion, reducing free radical formation, inhibiting calcium-dependent pathways in the early phase of IRI, and interfering in several intracellular signaling pathways, including NFkB.[9] We recently optimized a mice intestinal IRI model and established a maximum tolerable warm ischemia time beyond which the systemic impact would lead to death.[10] This model provides an alternative, which is to develop strategies aiming to ameliorate the IRI and secondary damage, resulting in survival. In the present study we used this model to evaluate the effect of IPC and TAC pretreatment as strategies to diminish intestinal IRI in mice. Although several studies were conducted to analyze the effect of both strategies as therapeutic options to attenuate IRI, no study combined TAC and IPC to prove any interactive effect between these two treatments. Therefore, the present study was designed to better understand the pathophysiology of IRI and to establish strategies to mitigate it.

MATERIALS AND METHODS

Animal Use and Care

Seventy adult male Balb/c mice (average weight, 25 ± 3 g) were housed in a climate-controlled room on a 12-hour light-dark cycle, fed with standard laboratory mice chow, and allowed water adlibitum. Mice were provided by the School of Veterinary Sciences of the National University of La Plata animal facility. All of the experiments were performed according to the guidelines set by the National Institutes of Health (NIH publication vol 25, no. 28, revised 1996).

Prior to the development of this protocol measurements of TAC plasma levels were performed by intragastric administration of TAC (3 mg/kg) 12 hours prior to blood sampling to evaluate drug absorption. TAC plasma levels showed an average of 10.6 ± 1.5 ng/mL.

Surgical Procedure

A model of intestinal IRI in mice by reversible occlusion of the superior mesenteric artery (OSMA) using a microvascular clamp was performed. Mice were anesthetized by i.p. injection of Ketamine (100 mg/kg) Midazolam (5 mg/kg), and Atropine (0.04 mg/kg). Lidocaine (10 mg/kg) was placed in the skin and subcutaneous cellular tissue as a local anesthetic. Postsurgical doses of morphine (2.5 mg/kg) were administered for pain control. The mice underwent celiotomy; intestinal loops were lateralized to the left flank. The superior mesenteric artery was isolated and occluded with a vascular clamp to induce intestinal ischemia.[10]

Experimental Groups

Fourteen animals were included in each group. In all groups except Sham (SH) intestinal ischemia was performed as described for a period of 40 minutes. Group 1; control (CT), OSMA was applied followed by reperfusion; Group 2; IPC, a 10-minute cycle of intestinal ischemia followed by ten minutes of reperfusion was performed before OSMA; Group 3, TAC intragastric TAC administration (3 mg/kg) was applied 12 hours before OSMA; Group 4, IPC + TAC, TAC was given 12 hours before IPC, performed as described in Group 2; at Group 5; SH, anesthesia, celiotomy, and superior mesenteric artery dissection without OSMA. Laparotomy was sustained for 70 minutes, accounting for the 40-minute intestinal ischemia in the other groups and 30-minute reperfusion.

Survival Analysis

Survival analysis was performed using six animals in each of the groups described (30 in all). After recovery on a thermal blanket they were returned to the facility to analyze survival by periodic observation. During the first 24 postsurgical hours, mice were observed every 3 hours. From the second to the seventh day, animals were evaluated once a day.

Histological and Molecular Assessment of IRI Damage

Evaluation of damage at the histological and molecular levels was performed using eight mice for each of the groups described above (40 mice in total). Mice were humanely killed by cervical dislocation 30 minutes after reperfusion and used for the following studies: postreperfusion, blood, lung, and intestinal samples. Due to the high sensitivity of the distal Jejunum to IRI damage, samples were obtained from this segment of the gastrointestinal tract.[11]

A portion of intestine and lung samples were fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. Sections were cut in a microtome and stained with hematoxylin-eosin. Intestinal samples were evaluated using Parkś score 0, normal mucosa; 1, subepithelial space at villus tip; 2, more extended subepithelial space; 3, epithelial lifting along villus side; 4, denuded villi; 5, loss of villus tissue; 6, crypt layer infarction; 7, transmucosal infarction; 8, transmural infarction.[12]

To determine remote damage, lung evaluation was based on the criteria established by Zhou et al.[13] Briefly, the parameters evaluated were as follows: 1, neutrophil infiltration; 2, interstitial edema; 3, airway epithelial cell damage; 4, hyaline membrane formation; and 5, hemorrage. Each parameter was scored (0, normal; 1, mild change; 2, moderate change; and 3, severe change). A general score resulting from the addition of each individual score was used to estimate lung damage. All samples were observed by 2 pathologists in a blinded way.

RNA Extraction, Reverse Transcription, and qPolymerase Chain Reaction

A portion of intestine was placed in lysis buffer and homogeneized immediately. Total RNA extraction was performed using Ilustra RNA Mini Extraction kit (GE Healthcare). Reverse transcription was performed using random primers and MMLV-reverse transcriptase (Invitrogen, United States). Real-time polymerase chain reaction (PCR) was performed following the manufacturer's protocol using the iCycler thermal cycler (BioRad, United States), IL-1b, and IL-6. Mouse b-actin was used for gene expression normalization. Relative difference calculation using the ΔCT method was previously described.[14]

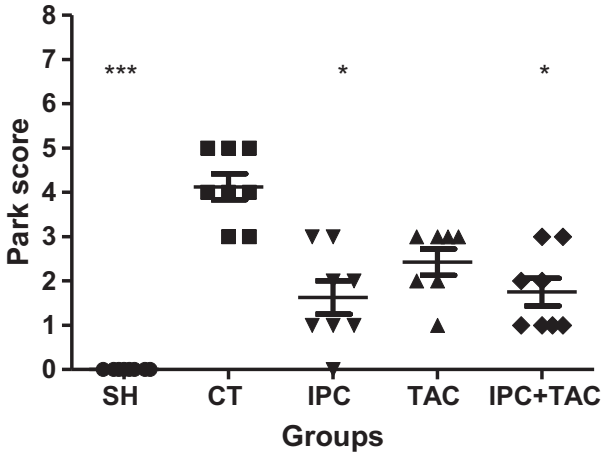

Fig 1. Different pretreatments modulate histological damage in the IRI intestinal murine model. Analysis of Park index of the jejunum of groups (n = 8) of mice receiving different pretreatments described in the Materials and Methods section. Each point represents an individual mouse. *$P < .05$ CT vs SH or IPC + TAC group. ***$P < .005$ (Kruskal-Wallis test).

For nitrites measurements, intestine samples were preserved in saline at −20°C. Nitrite measurements were performed using spectrophotometric method according to the technique described by Miranda et al and modified by Beda and Nedospasov.[15,16]

Statistics

Continuous variables were analyzed using 1-way analysis of variance, (ANOVA), followed by Dunnet post test. Discrete variables were analyzed using Kruskal, Wallis test. Survival curves were compared using log-rank test. All of the statistical analyses were performed using GraphPad software version 5.00 (San Diego, Calif, United States).

RESULTS

Histological Damage

Intestinal damage was scored according to Parks classification. The SH group showed a normal jejunum (Park 0). In the CT group, histological analysis showed an average of 4.1 ± 0.8 with a median of 4 (Fig 1). In this group, the most common observations were denuded or complete loss of villi. The IPC group had an average of 1.6 ± 1.1 with a median score of 1.5. The TAC group showed an average of 2.5 ± 0.7 (median 3). Park 3 was the most characteristic finding in the TAC group, showing enterocyte erosion in 5 of the 8 samples obtained. Finally, the IPC + TAC group had an average of 1.7 ± 0.8 with a median score of 1.5. In this group 50% of the samples showed an index of Park 1, characterized by the presence of edema limited to the tip of the villus. Statistical differences were observed between the CT versus the IPC + TAC group and the CT versus the IPC group ($P < .05$); however, no significant difference was observed between the CT and the TAC group.

Postsurgical Survival

All of the animals in the CT group died within 24 postreperfusion hours (3 between 15 and 18 hours and the remaining between 21 and 24 hours). All mice from SH, IPC, and IPC + TAC survived 7 days. In the TAC group, 4 mice reached a week survival and two animals died between 21 and 24 postreperfusion hours (Fig 2). Intestinal samples were taken randomly from animals that survived 7 postsurgical days and histological analysis was performed. As expected, all samples showed an index of Park 0 (data not shown).

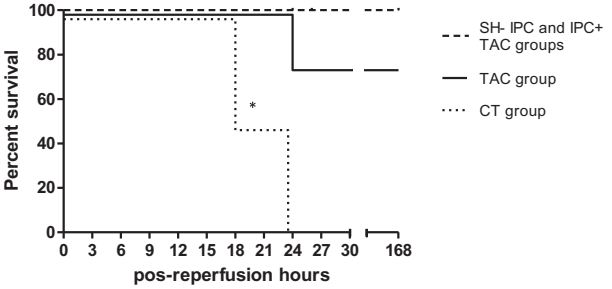

Fig 2. Pretreatment promotes survival in the IRI intestinal murine model. Survival curves of different groups (n = 6) as shown in Figure 1. All the animals underwent 40 minutes of intestinal ischemia and were given different treatments as stated in the Material and Methods section. Log-rank test for survival analysis. *$P < .002$ CT vs SH, IPC, TAC, and IPC + TAC groups.

Gene Expression in the Intestine

IRI has a differential impact on intestinal gene expression considering the analyzed markers. A high increase in gene expression of IL-6 and IL-1b was observed after 30 minutes of reperfusion of approximately 400- and 30-fold, respectively (Fig 3A and 3B). TAC or the combination of TAC + IPC down-regulated the expression of IL-6 and IL-1b ($P < .05$) more than 10-fold when compared with the CT group.

Nitrite Levels in Intestinal Tissue

IRI induced an increase in intestinal nitrite levels (Fig 4); the CT group showed the highest levels. Significant differences were observed between the CT group versus any of the pretreated groups ($P < .01$). The 54 group showed nitrite levels comparable with the pretreated groups.

Lung Histology

Microscopic evaluation of the lungs 30 minutes after reperfusion showed normal parenchyma in the SH group (Fig 5). Lungs from CT showed significant changes, showing a score of alteration with a median of 6 and an average of 5.8 ± 1.5. Moderate neutrophil infiltration, mild interstitial edema, airway epithelial cell damage, and hemorrhage were the most characteristic damage in this group. Microscopic findings in the IPC group (2 ± 1.5; median, 1) revealed minor alterations, with mild neutrophil infiltration in all samples as a characteristic finding. Within this group, only two lungs presented moderate neutrophil infiltration accompanied by mild edema and hemorrhage. The TAC group (2.5 ± 1; median, 3) showed mild edema and

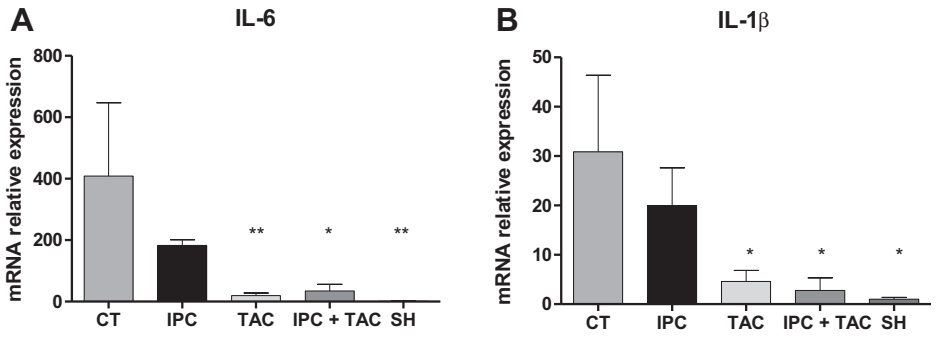

Fig 3. Pretreatment modulates intestinal proinflammatory gene expression. Relative mRNA expression levels of **(A)** IL-6 and **(B)** IL-1b on jejunum samples. In all cases, results were expressed relative to the average value of the SH group (n = 8). A significant difference (*$P < .05$; **$P < .01$) was determined using 1-way ANOVA followed by Dunnet post-test to compare each group against the CT group.

mild-moderate neutrophil infiltration without epithelial cell damage and hemorrhage. Minimal changes were present in lungs from the IPC + TAC group with an average of 1.2 ± 0.4 and a median of 1. Apart from 54, the latter group was the only one among the pretreated groups showing significant differences versus CT ($P < .05$).

DISCUSSION

Among the abdominal organs, the intestine is probably the most sensitive to IRI, a phenomenon causing morbidity and mortality in several intestinal diseases.[17] Therefore, establishing strategies to mitigate IRI and improve postsurgical survival are major aims for basic and translational research in the field of intestinal surgery including transplantation. Several experimental animal models have proven beneficial effects of IPC in intestinal IRI.[2,18] However, the present study is the first to evaluate the combination of IPC with inductive immunosuppression in mice. We have established a model that provides useful information to establish the relative contribution of each preventive strategy to be used in experimental intestinal surgery or transplantation, when IRI constitutes a major threat. Our results indicate that each pretreatment has a major impact in different parameters studied such as histological integrity or proinflammatory gene expression.

The IPC phenomenon has been observed in different organs, and several mechanisms may contribute to protection from IPC-induced IRI. Among them, decreased ATP consumption, reduced glycolysis, lower energy demand, mitochondrial integrity preservation, and reduced free oxygen radical production together with the activation of endothelial nitric oxide synthase reduce the degree of IRI and the associated remote effects.[19,20] In the intestine, IPC contributes to preservation of barrier function, diminishing the noxious effects of bacterial translocation. Recent publications disclose that IPC causes blood neutrophil priming, elevates production of superoxide and hydrogen peroxide on stimulation, and increases membrane translocation of cytosolic p47phox and p67phox as well as augmented bacterial-killing and phagocytotic activities.[21–23]

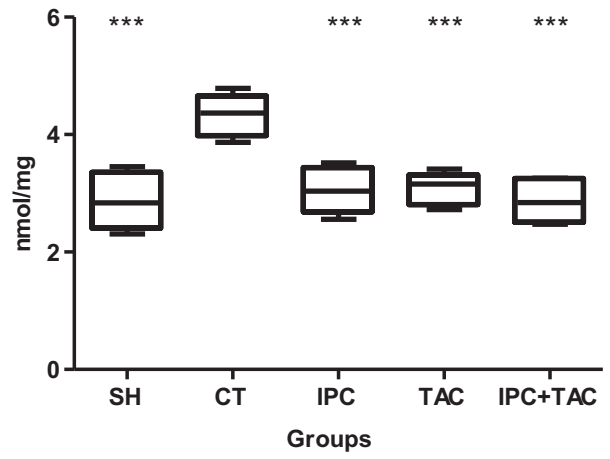

Fig 4. Pretreatment modulates nitrite production after intestinal IRI. Intestinal nitrite levels expressed in nmol/mg. Significant differences in the CT group compared with treated and SH groups were determined using 1-way ANOVA. ***$P < .01$.

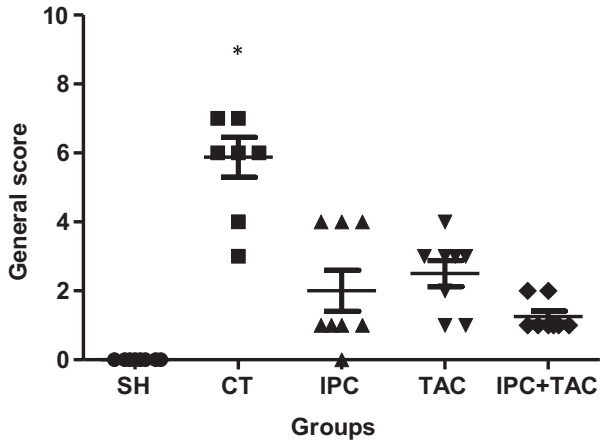

Fig 5. Pretreatment modulates remote organ damage in intestinal IRI. Histological lung damage in each group (n = 8) was calculated as a score of different architectural and cellular features as described in the Materials and Methods section. Significant differences were observed in the CT group vs IPC + TAC treatment. *$P < .05$ (Kruskal-Wallis test).

Our results underline the important impact of IPC on the murine intestine; it preserves the integrity of the intestinal histology and improves animal survival compared with animals that underwent pharmacological preconditioning. All the animals that were mechanically preconditioned evidenced histological improvement compared with pharmacological preconditioning alone.

It has been reported that immunosuppressive donor pretreatment in a murine model may attenuate IRI. Cicora et al demonstrated that TAC and Rapamycin pretreatment reduced necrosis, reduced apoptosis, and improved clinical outcomes in a rat syngenic kidney transplantation model.[24] Thymoglobuline was also used as a predonor treatment strategy, and improved IRI in kidneys, reducing delayed graft function and improving survival.[25]

Our study stresses the correlation between intestinal histological damage and survival rate. All animals presenting Park grade less than 3 exhibited a long survival rate, but on the other hand all mice with Park values 4 or greater died. Animals with Park 3 may either survive or die, probably depending on the extension of the Park 3 lesions because the histology usually shows the worst finding and the biopsy only represents a small segment of the intestine, thus the outcome might be a surrogate of tissue injury severity and extension. This is in agreement with our previous results in intestinal transplantation in rats, where all CT animals with Park scores greater than 3 died within 24 postreperfusion hours.[26] On the other hand, most of the treated mice survived, except for 2 animals pretreated with TAC that died within 21 and 24 postreperfusion hours, respectively (Fig 2). In the histological analysis, this group showed the second highest Park score after the CT group (Fig 1).

It has been reported that donor pretreatment with TAC reduces NFkB pathway activation reducing inflammation, tissue destruction, and remote organ damage.[9] Transplantation of preconditioned intestinal grafts is associated with lower inflammatory activation and remote organ injury in rats.[27] We used a proinflammatory cytokine panel to evaluate the degree of early tissue activation upon IRI. IL-6 and IL-1b are a triad of cytokines involved in inflammation and innate response activation triggered by tissue damage.[25] Our results indicate that pretreatment with TAC plays an important role in the intestinal early, messenger RNA (mRNA) expression of proinflammatory cytokines, such as IL-6 and IL-1b, whereas IPC affects the expression of these cytokines only marginally. Although TAC alone significantly reduces the expression of IL-6 and IL-1b compared with controls (Fig 3) in our murine model it fails to improve histological injury and survival to the same degree as IPC or IPC + TAC (Fig 1 and Fig 2).

As observed in the experiments performed in rats by Wang et al[28] cytokines fail to predict IPC protection against IR damage. Local production of the cytokines measured did not correlate with tissue damage and survival because the IPC group showed high expression of IL-6 and IL-1b with low histological damage and 100% survival.[29] We have not detected early local expression of TNFa (data not shown), indicating that this cytokine might not be involved in the initial events of IRI pathogenesis, as previously suggested.[30,31]

Inducible NOS activation has been proposed as an important player in intestinal IRI[32,33]; IPC has been described as a mechanism to prevent intestinal IRI by inhibition of NOS. Nitrite production is a rough measurement of reactive nitrogen species, mainly by activation of intestinal inducible NOS due to IRI.[34] In our study a good correlation of this indicator with tissue damage was observed, showing the protective effects of the different pretreatments used.

We have observed that remote organ damage occurs as early as 30 minutes after reperfusion and with high correlation with intestinal tissue damage. It has been proposed that remote organ innate recognition of translocated microbiota and/or intestinal luminal microbial products is a major contributor to this phenomenon, regardless of intestinal cytokine production.[35] Since differences in lung histology were observed for combined IPC + TAC, presumably a role for proinflammatory cytokines may be postulated. In this group, a lower expression level of IL-6 and IL-1b was observed as compared with IPC alone; however, in both cases, intestinal histology was preserved in the same degree. Taken together, these observations may indicate that the effects of lung histology may be attributed to the combination of both signals derived from the loss of intestinal barrier integrity due to damage and systemic increase of proinflammatory mediators, which were not assessed in our study.

In mice, the use of mechanical or pharmacological IPC opens a variety of possibilities depending on the model to be evaluated, eg, if researchers are planning to reduce IRI in a non-transplantation model IPC will be the appropriate option. Moreover, if a transplantation model is the aim, authors can choose between pharmacological or combined (IPC + drugs) therapy based on the primary experiment. This study is the first to depict IPC evaluation in mice and the first to combine IPC and TAC pretreatment as strategies to ameliorate intestinal IRI. These simple procedures combined ameliorate local intestinal IRI and decrease the production of pro-inflammatory cytokines, nitrite levels, and intestinal tissue damage. The local decrease of IRI is reflected at systemic levels, showing less lung injury and improving the survival rate in the groups treated as compared with the control group. The use of mechanical preconditioning alone could be easily translated to the clinical setting of intestinal surgery or transplantation to minimize the deleterious effect of IRI in humans. A combination of both strategies might be used in the transplantation setting. The use of other immunosuppressive drugs, such as thymoglobulin or sirolimus, will be included in our future research projects.

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