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**DOTTORATO DI RICERCA IN FARMACOLOGIA E TOSSICOLOGIA  
SPERIMENTALI**

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**Investigation into the  $\mu$  opioid receptor  
role in an experimental model of  
mesenteric ischemia/reperfusion.**

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## ABSTRACT

Intestinal ischemia is a clinical gastrointestinal emergency associated with high morbidity and mortality. Aim of this study was to investigate whether activation of  $\mu$  opioid receptors ( $\mu$ ORs) protects from the injury induced by intestinal ischemia and reperfusion. Ischemia was induced by occlusion of the superior mesenteric artery (45 min) and followed by reperfusion (5hours) (I/R). Sham Operated (SO) and normal (N) mice served as controls. Each group received subcutaneously: (1) saline solution; (2) the  $\mu$ OR selective agonist, [D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin (DAMGO) (0.01 mg kg<sup>-1</sup>); (3) DAMGO and the selective  $\mu$ OR antagonist [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>] (CTAP) (0.1 mg kg<sup>-1</sup>) or (4) CTAP alone. Compared to SO, I/R induced intestinal inflammation as indicated by the histological damage and the significant increase in myeloperoxidase (MPO) activity (index of tissue neutrophil accumulation). Treatment with DAMGO significantly reduced tissue damage and MPO activity in I/R and these effects were reversed by CTAP. Significant increase in levels of the inflammatory cytokine TNF- $\alpha$  and IL-10 mRNA was detected in I/R mice compared to SO. DAMGO significantly reduced TNF- $\alpha$  mRNA levels in I/R, effect that was abolished by CTAP, but did not modify IL-10 mRNA levels.  $\mu$ OR mRNA levels were comparable in I/R, SO and N mice. There was a significant delay in gastrointestinal transit in both I/R and SO compared to N mice, which was not affected by DAMGO administration. In summary, this investigation shows that exogenous activation of  $\mu$ ORs plays a protective role in our experimental model of mesenteric I/R in mice. Indeed,  $\mu$ OR activation is able to reduce the intestinal damage induced by I/R, through a mechanism that appears to be in part mediated by TNF- $\alpha$ .

*Alle mie famiglie:  
italiana ed americana...*

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# INTRODUCTION

# 1. INTESTINAL ISCHEMIA AND REPERFUSION

## 1a. PATHOGENESIS AND PHYSIOLOGY

Intestinal ischemia and reperfusion injury could occur in several clinical situations such as abdominal surgery, small bowel transplantation, cardiopulmonary bypass, strangulated hernias and neonatal necrotizing enterocolitis.<sup>1</sup> Intestinal ischemia is a real gastrointestinal emergency, characterized by high morbidity and a mortality rate ranging from 60% to 80%, depending on the source of obstruction<sup>2</sup>. During this pathology the three arteries usually compromised by obstruction are the celiac trunk, the superior mesenteric artery (SMA) and the inferior mesenteric artery (IMA). Recently, the American Gastroenterology Association (AGA) classified this severe abdominal event into three major categories, focusing on clinical features:

1. *Acute mesenteric ischemia*: further subdivided into major arterial occlusion, minor arterial occlusion, major embolus, mesenteric venous thrombosis or splanchnic vasoconstriction (non-occlusive mesenteric ischemia) based on the etiology of the occlusion.
2. *Chronic ischemia*: consequence of longstanding atherosclerotic diseases and also known as intestinal angina.
3. *Ischemic colitis*: correlated to colonic ischemia.<sup>3,4</sup>

Patients which develop acute embolic ischemia often have a history of cardiac diseases, like atrial fibrillation and ventricular aneurysm. Thrombotic ischemia could be a consequence of both venous and arterial thrombosis and is usually related to a history of atherosclerotic disease and chronic abdominal pain. In the non-occlusive

patients no vascular occlusion is usually demonstrated because blood flow is present in larger arteries, but ischemia is consequence of persistent vasospasm correlated to severe cardiac failure, sepsis, vasoconstriction induced by renin, angiotensin, vasopressin, thromboxanes and leukotrienes or administration of  $\alpha$ -adrenergic agents and medications (i.e. digitalis)<sup>2</sup>.

The most common cause of chronic ischemia is atherosclerotic occlusion or severe stenosis of mesenteric arteries. Slow-growing obstruction into the vessel brings to a gradual reduction in blood flow to the intestine and since collateral circulation is present the patient may be asymptomatic even with stenosis of several arteries. Chronic mesenteric ischemia usually causes abdominal pain and weight loss.<sup>3</sup>

Ischemic colitis represents the consequences of acute or, more commonly, chronic blockage of blood flow through arteries that supply the large intestine. Patients experience abdominal pain, usually, localized to the left side of the abdomen, along with tenderness and bloody diarrhea. Severe ischemia may lead to bowel necrosis and perforation, which results in an acute abdomen and shock, frequently, being accompanied by lactic acidosis<sup>5</sup>.

During the ischemic insult interruption of blood supply leads to poor organ oxygenation which rapidly damage metabolically active tissues. Even a short period of ischemia can impair mucosal functions, whereas a severe and longer ischemia can cause transmural necrosis of the bowel wall with perforation and peritonitis.

At the cellular levels, ischemia causes mitochondrial dysfunction by blocking aerobic energetic metabolism. The depletion of intracellular levels of adenosine triphosphate (ATP) alters cellular homeostasis generating loss of ion transfer regulation, intracellular acidosis, changes in membrane permeability and fluidity which leads to intracellular

accumulation of ions accompanied by an influx of water and swelling of cells<sup>6</sup>. Endothelial cells lose their anti-adhesive properties and develop thrombogenic and adhesive surface. In the main time, progressive accumulation of toxic metabolites, diminished production of bioactive agents (e.g. nitric oxide, prostacyclines) and release of degradative enzymes lead to cell death and tissue necrosis.<sup>7</sup>

During reperfusion, restoration of blood flow and reintroduction of oxygen in the ischemic tissue initiate a cascade of events that may exacerbate cell injury (oxygen paradox) and this reperfusion damage is frequently more severe than the initial ischemic insult. The renewed presence of oxygen leads to oxidative stress with formation of cytotoxic reactive oxygen species (ROS), release of pro-inflammatory substances and activation and adhesion of polymorphonuclear neutrophils (PMNs). This adhesion of leukocytes to the endothelial surface and consequent activation impairs endothelial cell layer integrity and allows leakage of proteins and macromolecules into extra-vascular space producing tissue edema. Moreover, activated neutrophils, endothelium, monocytes and platelets release ROS and a variety of inflammatory cytokines, such as tumor necrosis factor  $\alpha$  (TNF-  $\alpha$ ), platelet activating factor (PAF) and leukotrienes generating a sort of vicious circle which enhances exponentially the inflammatory response.

Potentially every organ can be subject to I/R damage, however, the intestine is one of the internal organ most sensible to I/R injury because enterocytes and endothelial cells are labile and particularly susceptible to damaging effects of both hypoxia (ischemia) and re-oxygenation (reperfusion).<sup>1</sup>



## **1b. MEDIATORS INVOLVED IN I/R INJURY**

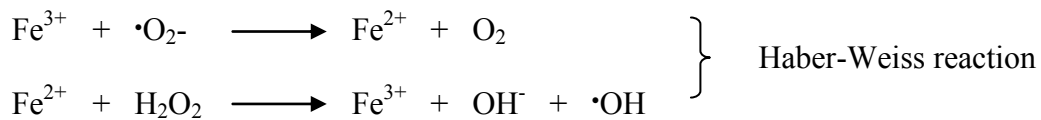
Intestinal ischemia-reperfusion injury is consequence of a complex cascade of events correlated to imbalance between oxygen consumption and supply, production of cytotoxic and reactive molecules and onset of acute inflammatory reaction. A multitude of mediators play a role in the development of I/R tissue damage, the mains are listed below.

### **Xanthine Oxidase and Reactive Oxygen Species**

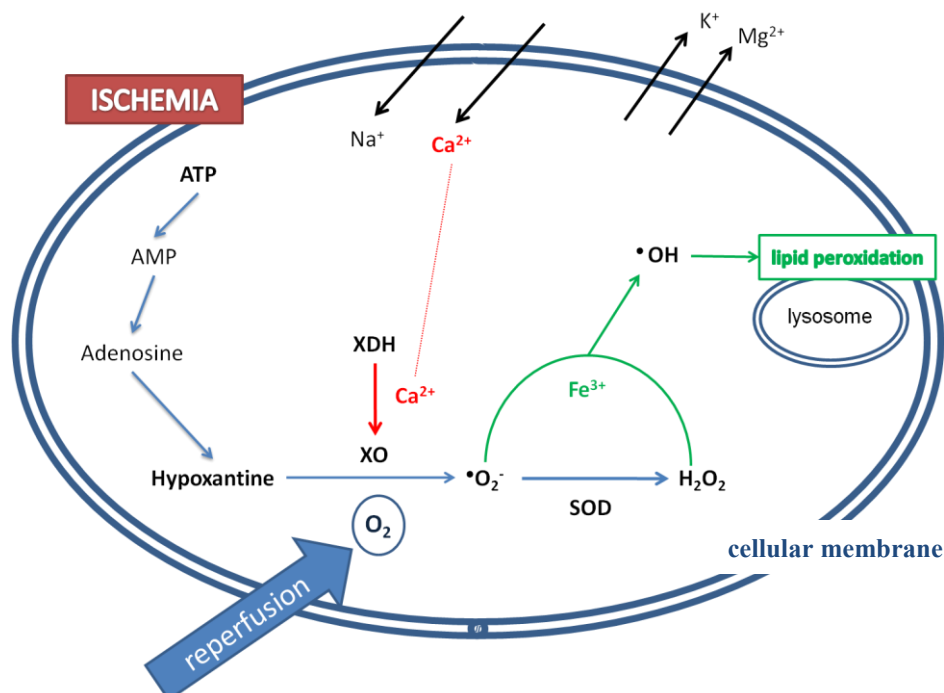
Reactive Oxygen Species (ROS) are extremely reactive and unstable free radicals (molecules which have a single unpaired electron in their orbit) originating when O<sub>2</sub> is reintroduced in the ischemic tissue during the reperfusion period. ROS can be involved in reaction with organic and inorganic chemical substances including proteins, lipids, carbohydrates and particularly cell membranes and nucleic acids generating severe cellular damage.<sup>7</sup>

During the ischemic period ATP is catabolized to adenosine and then to hypoxanthine. Under physiological condition hypoxanthine is converted to xanthine by Xanthine Dehydrogenase (XDH). During ischemia the depletion of ATP creates loss of ATP-dependent ion channel regulation which results in passive ions shift across cell membranes: K<sup>+</sup> and Mg<sup>2+</sup> diffuse out, but Na<sup>+</sup>, Ca<sup>2+</sup> and H<sub>2</sub>O diffuse in, causing cell swelling.<sup>1</sup> Accumulation of intracellular Ca<sup>2+</sup> enhances conversion of XDH to Xanthine Oxidase (XO), with degree of conversion proportional to the length of ischemic period. During reperfusion O<sub>2</sub> reintroduced into the tissue reacts with hypoxanthine and XO to produce the superoxide anion ( $\cdot\text{O}_2^-$ ). This kind of oxygen free radical, under physiological conditions, is transformed into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by the action of

the enzyme Superoxide Dismutase (SOD) and its damaging effects are prevented. However, during reperfusion of ischemic tissue, these natural defenses may be overcome. Moreover, in presence of iron ( $\text{Fe}^{3+}$ ),  $\text{H}_2\text{O}_2$  and  $\cdot\text{O}_2^-$  undergo the Haber-Weiss reaction and produce hydroxyl radical ( $\cdot\text{OH}$ ) which is more reactive and cytotoxic than the relatively low-energy radical  $\cdot\text{O}_2^-$ .



$\cdot\text{OH}$  is able to react with the fatty acid side chain of the membrane phospholipids generating lipid peroxides, that are themselves free radicals. Consequence of this lipid peroxidation is a severe damage at organelles and cell membranes, with loss of selectivity in ionic exchange and release of hydrolytic enzymes and cytotoxic products responsible for cell necrosis and apoptosis.<sup>7</sup> ROS and substances released from the cells are also able to attract and promote the adherence of granulocytes to the microvascular endothelium.



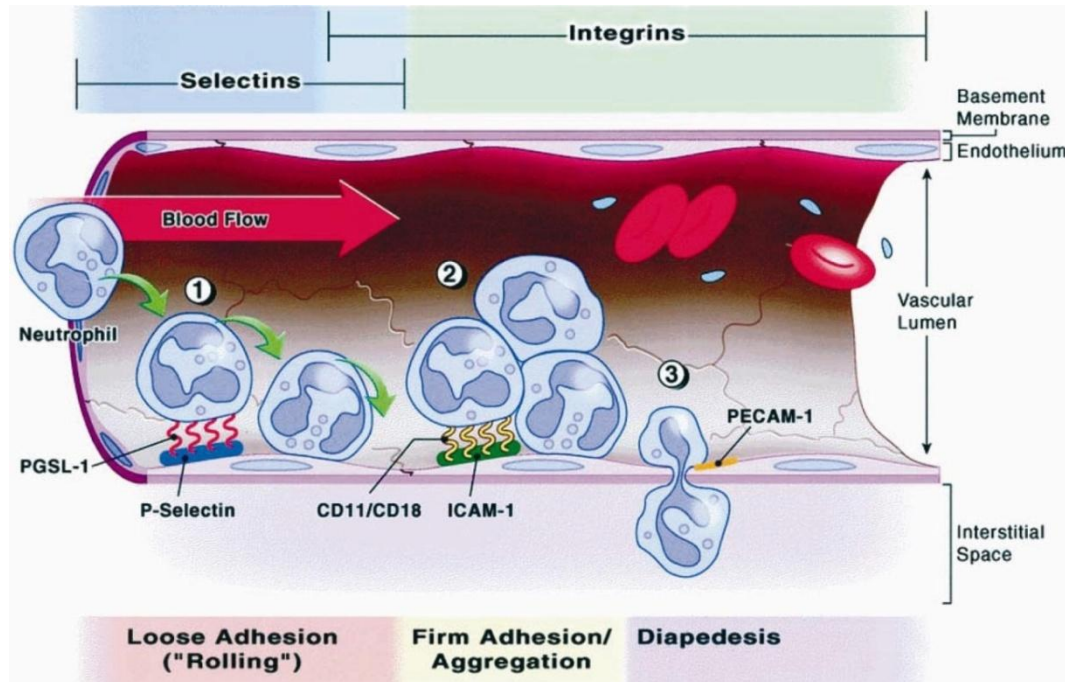
**Figure 1:** Schematic mechanism of cellular injury induced by Reactive Oxygen Species (ROS) during I/R. During ischemia ATP intracellular store are depleted while hypoxanthine is accumulated. During reperfusion  $\text{O}_2$  react with Xanthine Oxidase (XO), Superoxide Dismutase (SOD) and iron generating ROS.

## **Polymorphonuclear Neutrophils (PMNs)**

Occlusion and reperfusion of an artery triggers a vigorous immune response. Acute ischemia causes activation of the endothelium with increased permeability and expression of adhesion molecules. Moreover the increased release of bioactive substances from the endothelial cells augments the recruitment and the infiltration of circulating leukocytes into the post-ischemic tissue. Following I/R injury, polymorphonuclear neutrophils (PMNs) have been shown to be the major leukocytes found in necrotic tissue, particularly in postcapillary venules<sup>8</sup>.

The process of migration of PMNs from the postcapillary venules to the area of inflammation is complex and highly coordinated. It can be subdivided in three phases:

1. *Rolling:* PMNs roll along the endothelium surface. This event is mediated by interaction between endothelial adhesion molecules (like P-selectine and E-selectine) and L-selectine which are constitutively expressed on leukocytes. During I/R on the endothelial cells there is an increased expression and/or avidity of these adhesion molecules.
2. *Adhesion:* rolling along the endothelial cell surface brings PMNs into close proximity to chemoattractants (like leukotriene B<sub>4</sub>, C5a, Interleukine-8 and PAF) and chemokines. Interaction of these molecules with leukocyte receptors activates  $\beta_2$ -integrins (CD11a, CD11b, and CD11c/CD18) which strongly bind intracellular adhesion molecules (ICAM-1) and platelet/endothelial cell adhesion molecules (PECAM-1) present on endothelial vascular cells.
3. *Extravasation:* the interaction between PMNs and endothelial cells allow the extravasation of the leukocyte out of the blood vessel and into the interstitial space.<sup>1</sup>



**Figure 1:** Process of migration of polymorphonuclear neutrophils from the venules to the area of inflammation. Mechanism of activation: rolling, adhesion and extravasation. (Reproduced from *Mallick et al., Dig Dis Sci 2004*)

Active PMNs after adhesion and migration through the endothelium generate local damage through release of a burst of free radicals, proteolytic enzymes (collagenase, elastase, and cathepsin) and peroxidase (myeloperoxidase). While XO appears to account for the initial oxidant stress elicited in venules after a few minutes of reperfusion, activated PMNs appear related to the greater oxidant stress that occurs thereafter. Neutrophils activation and infiltration increases the vascular permeability triggering endothelial barrier dysfunctions. Moreover PMNs can cause tissue injury by physically impairing microcirculation and thereby increasing the extension of the ischemic area.

Summarizing, activated neutrophils contribute to intestinal ischemia-reperfusion injury through multiple mechanisms: release of free radicals and proteolytic enzymes as well as stimulation of cytokine release from local cells; thus promoting further neutrophil recruitment and plugging of capillaries.

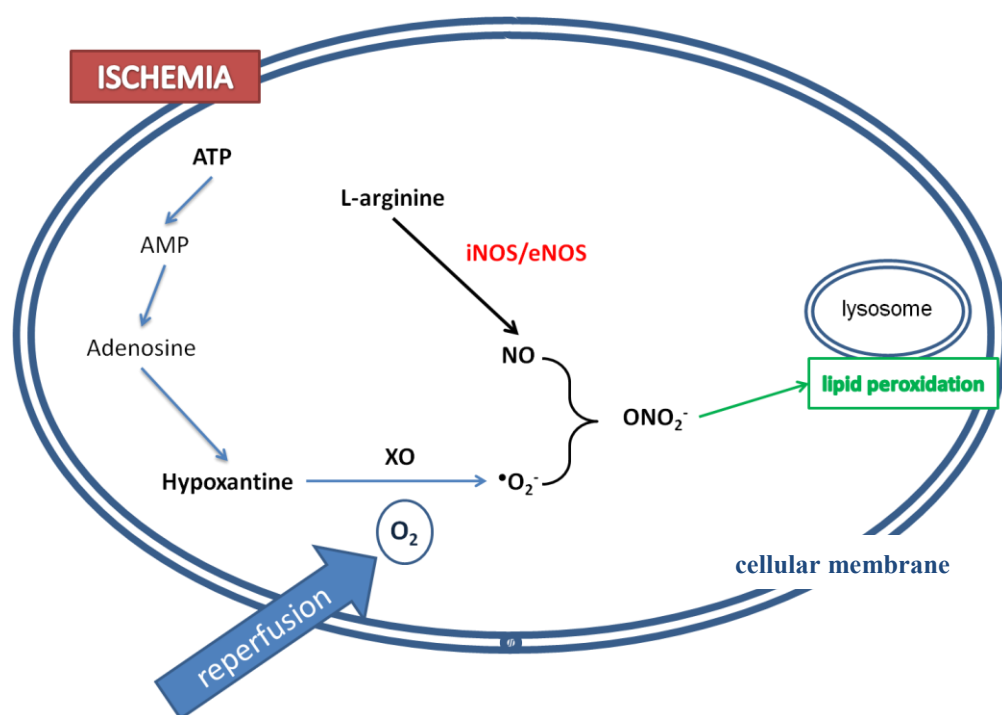
## Nitric oxide

Nitric oxide (NO) is a ubiquitous biological messenger involved in many physiological and pathophysiological events. NO is a free radical highly reactive synthesized via oxidation of the guanidinic group of L-arginine by a family of NO synthase enzymes (NOS), which three isoforms have been identified and cloned: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS)<sup>9</sup>. NO produced by constitutively expressed eNOS contributes mainly to the regulation of blood pressure, organ blood flow distribution and platelets aggregation. NO produced by nNOS has an important role as neurotransmitter both in the brain and in the gastrointestinal tract. iNOS expression is induced only in response to cytokines, growth factors and endotoxins produced by a variety of cells (e.g. smooth muscle cells and macrophages)<sup>10</sup>.

NO plays a double role in intestinal I/R, either protective or toxic. Under normal condition NO, produced in small amount, has various important physiological roles like, for instance, to reduce arteriolar tone via guanylate cyclase activation in smooth muscle, to inhibit platelet aggregation, to minimize adhesive interactions between leukocytes and mesenteric endothelium surface and to prevent formation of substances that activate NF-kB. Another important role of NO is to scavenge the intracellular levels of ROS; particularly it reacts with the superoxide anion ( $\cdot\text{O}_2^-$ ) producing peroxynitrite ( $\text{ONO}_2^-$ ): a molecule that is not itself a free radical, but is a powerful oxidant that can cause pronounced lipid peroxidation when present in relevant concentration.

Under physiological conditions the flux of NO largely exceeds the  $\cdot\text{O}_2^-$  production, and NO is able to exert its benefic effects on intestine tissue, however, during I/R, NO could be involved in the production of cytotoxic molecules. During the early phase of reperfusion there is an accumulation of  $\cdot\text{O}_2^-$  and an augmented production of NO

through activation of iNOS. These concomitant events enable almost all the NO produced reacts with the huge amount of  $\cdot\text{O}_2^-$  present, leaving little or no bioactive NO available to maintain optimal tissue blood flow and to minimize interaction between endothelial cells, platelets and immune cells (particularly PMNs). Loss of endogenous NO has disastrous consequences resulting in severe dysfunction of the endothelium and the presence of  $\text{ONO}_2^-$  implies augment of the already strong oxidative stress, thus contributing to exacerbate the I/R injury.



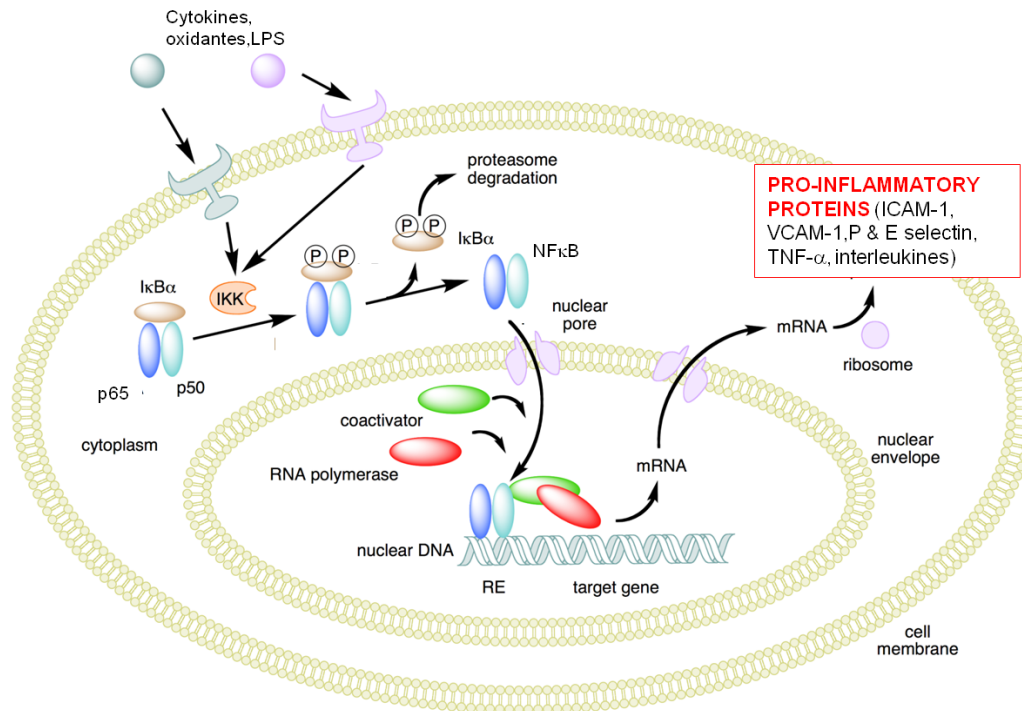
**Figure 2:** Schematic mechanism of cellular injury induced by NO derivatives during I/R. NO reacts with radical oxygen species, generated during reperfusion, producing peroxynitrite ( $\text{ONO}_2^-$ ) which induces peroxidation of membrane phospholipids.

### **Nuclear Factor $\kappa$ -light-chain-enhancer of activated B cells (NF- $\kappa$ B)**

NF- $\kappa$ B is a specific nuclear transcription factor which regulates the expression of pro-inflammatory genes and appears to perform an important function in generation and resolution of I/R injury.

NF- $\kappa$ B is activated in the intestine by inflammatory stimuli (e.g. PAF and leukotriene B<sub>4</sub>), cytokines (e.g. TNF- $\alpha$ , IL-1 $\beta$ ), LPS and oxidants, and the activation of this transcription factor leads to expression of various genes, codifying different proteins involved in the amplification and maintenance of the inflammatory response.

NF- $\kappa$ B is a member of dimeric transcription factor complex families. In the cytoplasm of unstimulated cells, it is normally found in its inactive form (p50/p65/I- $\kappa$ B $\alpha$  ternary complex). The complex formed by NF- $\kappa$ B and I $\kappa$ B $\alpha$  coordinates the activation of a series of genes codifying for cytokines and adhesion molecules associated to I/R events. Stimulation of cell by vast number of agents, including cytokines (such TNF- $\alpha$  and IL-1 $\beta$ ) free radicals, oxidants or inflammatory mediators results in phosphorylation and ubiquitination of the I- $\kappa$ B $\alpha$  inhibitory subunit, which then undergoes to proteasome degradation. The active complex p50/p65 is now able to translocate into the nucleus and to activate transcription of its target genes, which are diverse and include those involved in the inflammatory response, cell adhesion and growth control. Indeed, NF- $\kappa$ B induces expression of a variety of pro-inflammatory proteins such as adhesion molecules (ICAM-1, VCAM-1, P- & E-selectine), tumor necrosis factor (TNF- $\alpha$ , - $\beta$ ), interleukins (IL-1, IL-6), chemokines and inducible enzymes (iNOS, COX-2)<sup>11</sup>.



**Figure 4:** Mechanism of NF- $\kappa$ B action. In an inactivated state, NF- $\kappa$ B is located in the cytosol complexed with the inhibitory protein I- $\kappa$ B $\alpha$ . A variety of extracellular signals can activate the enzyme I $\kappa$ B kinase (IKK), which phosphorylates the I $\kappa$ B $\alpha$  protein, which results in ubiquitination and dissociation of I $\kappa$ B $\alpha$  from NF- $\kappa$ B. The activated NF- $\kappa$ B is then translocated into the nucleus and begins the transcription of genes codifying pro-inflammatory proteins. (modified from Wikipedia)

During the reperfusion period, re-oxygenation appears to be the key stimulus for activation of the I- $\kappa$ B kinase (IKK) responsible for the phosphorylation of the inhibitory subunit and consequent activation of NF- $\kappa$ B.<sup>12</sup> Inflammatory response elicited by I/R occurs several hours after the beginning of reperfusion due to this transcription-dependent synthesis of pro-inflammatory proteins.

Activation of the NF- $\kappa$ B family of transcription factor has been demonstrated in various models of experimental I/R, including intestinal I/R. *In vivo* studies on mice lacking IKK, investigated the role of NF- $\kappa$ B in controlling I/R injury and showed that inhibition of NF- $\kappa$ B activation in enterocytes prevents the increase in systemic TNF- $\alpha$  concentration after I/R and consequently reduces systemic inflammatory response. In the main time, inhibition of NF- $\kappa$ B activation also resulted in severe apoptotic damage



to the reperfused intestinal mucosa, suggesting a dual function of NF- $\kappa$ B system, responsible for both tissue protection and systemic inflammation<sup>13</sup>.

### **Cytokine cascade**

The inflammatory cascade triggered by I/R events results in the secretion of multiple pro-inflammatory and counter-regulatory cytokines that play a pivotal role in I/R induced destruction of intestinal mucosa, tissue injury, systemic toxicity and lethality.

Among the whole set of cytokines, previous studies performed in rodents have shown the involvement of TNF- $\alpha$ , IL-1 $\beta$  and IL-10 in modulating intestinal I/R damage. During I/R insult, TNF- $\alpha$  is amply released from macrophages, monocytes, lymphocytes and mast cells and elicits leukocyte migration, fever and acute-phase of the inflammatory response. This pro-inflammatory cytokine plays an essential role in enhancing neutrophil influx, tissue injury and lethality in experimental models of I/R. Indeed, treatment of rats with anti-TNF- $\alpha$  antibodies prevented neutrophil influx and tissue injury after intestinal I/R<sup>14</sup> and treatment of mice with a soluble chimeric form of the TNF- $\alpha$  receptor reduced lethality correlated with greater and earlier TNF- $\alpha$  increase in serum<sup>15</sup>.

Interleukin 1 $\beta$  (IL-1 $\beta$ ) is a cytokine released from macrophages and other antigen-presenting cells, which promotes the inflammatory response by increasing endothelial leukocyte adhesion and phagocyte activation. IL-1 $\beta$  has also been shown to activate the IL-8 gene thereby increasing the neutrophil population at the site of inflammation. Serum levels of interleukin 1 $\beta$  significantly increase after I/R<sup>16</sup> and play a pivotal role in the systemic inflammatory response syndrome (SIRS), which can lead to sepsis and multisystem organ failure if not corrected<sup>17</sup>.

Several studies have now demonstrated that IL-10 modulates pro-inflammatory cytokine production and tissue injury following ischemia and reperfusion injury. IL-10 normally serves to down-regulate the inflammatory response, but its role in I/R injury is controversial. Indeed, studies in IL-10 deficient mice suggested that endogenous IL-10 exerted anti-inflammatory role during reperfusion injury, possibly by regulating early stress genetic response (c-Jun kinase activation), neutrophil influx and cytokine production<sup>18</sup>. Exogenous administration of IL-10 also reduced the systemic inflammatory response in a rodent model of intestinal reperfusion injury, an effect associated with inhibition of cytokine production and neutrophil accumulation<sup>19</sup>. Other studies on mice demonstrated that this anti-inflammatory cytokine does not play a significant local or systemic protective role in I/R injury<sup>20</sup>, or even that IL-10 may increase the tissue damage after intestinal I/R<sup>21</sup>. These contrasting effects could be referred to different involvement of this cytokine during the time-course of reperfusion phase; anyhow further studies are required to clarify the role of IL-10 in I/R damage.

## **Complement**

The complement system is part of the immune system and consists of a number of small proteins found in the blood, normally circulating as inactive zymogens. When stimulated by one of several triggers, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. The result of this activation cascade is a massive amplification of the immune response and activation of the cell-killing membrane attack complex.

Several studies showed that activation of complement system is responsible for I/R injury of the intestine.<sup>1</sup> In fact, reperfusion is able to activate the complement cascade leading to widespread deposition of complement fragments on the microvasculature. These complement activation products are chemotactic for leukocytes and neutrophils, elicit oxidant formation and are also able to induce expression of adhesion molecules genes (e.g. ICAM-1) and up-regulation of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1  $\alpha$ , INF- $\gamma$ ). Moreover, activation of complement system can lead to translocation into the nuclei of NF- $\kappa$ B and consequent increased transcription of a variety of pro-inflammatory proteins and enzymes (e.g. iNOS, interleukins, adhesion molecules)<sup>22</sup>.

## **T-cells and B-cells**

It is well established that the innate immune system, such as complement, PMNs, cytokines and macrophages participate in ischemia reperfusion injury. Recent evidence showed that also lymphocytes and cell-mediated immunity play an important role in pathophysiology of I/R damage<sup>8</sup>.

Indeed, studies on mice with severe combined immunodeficiency (SCID), lacking both T and B cells, gave evidence that intestinal I/R-induced leakage of albumin is associated with the recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. Moreover, T-cells seem to modulate the recruitment of neutrophils that occurs hours after reperfusion, and this effect could account for the increased endothelial permeability that occurs within minutes after reperfusion rather than direct cytotoxic actions of the T-cells themselves<sup>23</sup>. Although most evidence has linked lymphocytes with the early I/R injury, emerging data associate these cells with healing from ischemic damage: CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells reduce the pro-inflammatory sequel of I/R by altering the chemokine and chemokine-receptor expression<sup>24</sup>.

While the role of T cells can be either pathogenic or protective depending on timing of the injury and type of T-cells, the role of B cells has been found to be predominantly pathogenic<sup>25</sup>.

## **Neurotransmitters, autacoids and enzymes**

A variety of neurotransmitters, autacoids and enzymes locally released could perform an important function in the pathogenesis of intestinal I/R injury. In the last years our group analyzed the involvement of different bioactive substances in an experimental model of mesenteric I/R in rodents.

Intestinal ischemia followed by 24 hours of reperfusion in rats increased the expression of the inhibitory transmitter vasoactive intestinal polypeptide (VIP) and, in the main time, reduced the expression of the excitatory transmitter substance P (SP). Observation that changes in these transmitter expression in I/R rats were prevented by pre-treatment with a NOS inhibitor [N-nitro-L-arginine, L-NAME], or a N-methyl-D-aspartate receptor (NMDA-R) antagonist [(+)-5-methyl-10,11-dihydro-5HT-[a,b]cyclohepten-5,10-imine, MK-801], suggests an involvement of the glutamatergic system and its interaction with nitric oxide in intestinal ischemia/reperfusion. We propose that glutamate released locally in response to I/R activates the NMDA-Rs thus triggering a sustained influx of  $Ca^{2+}$  into neurons leading to NOS activation and NO production and adaptive changes in enteric transmitters that might contribute to gastrointestinal dysmotility and local inflammatory responses induced by I/R<sup>26</sup>.

Serotonin (5-HT) is an important neurohumoral transmitter widely distributed at both central and peripheral level (80% of total body 5-HT is found in the gut, predominantly within the granules of mucosal enteroendocrine cells). Previous study on rats showed an increase in the plasmatic levels of serotonin after mesenteric I/R, probably due to an increase in the release of 5-HT by the injured intestine<sup>27</sup>. Different findings support either negative or protective role for 5-HT in intestinal I/R conditions, depending on

which receptor subtypes are activated. Through administration of selective agonist [DOI: (1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane)] or antagonist [ketanserin] of the 5-HT<sub>2A</sub> receptor subtype, we investigated the role of this receptor in the functional alterations and inflammatory response caused by mesenteric I/R in mice. We showed that inhibition of 5-HT<sub>2A</sub> receptors protects against mesenteric I/R injury, preventing gastrointestinal transit delay as well as tissue edema and leukocytes infiltration<sup>28</sup>.

Reperfusion of ischemic tissue results in increased production of oxidants and radicals that in turn cause mast cells activation and degranulation, release of enzymes and bioactive amines (e.g. histamine)<sup>29</sup>. Such event has been recognized as promoter of inflammatory cells infiltration, increased vascular permeability and mucosal dysfunction induced by mesenteric I/R but, so far, there is no clear indication about the protective or deleterious effect played by histamine (H) during episodes of intestinal hypoxia and re-oxygenation. We assessed the effects evoked by different histamine receptor (HR) subtypes on motor disorders and inflammation induced by I/R in mice, through the administration of selective histamine receptor blockers [H<sub>1</sub>R: mepyramine, H<sub>2</sub>R: famotidine, H<sub>3</sub>R: M85 or H<sub>3</sub>R/H<sub>4</sub>R: thioperamide]. Beneficial effects of selective histamine H<sub>2</sub> and H<sub>4</sub> receptors blockade were observed: famotidine prevented I/R-induced colonic transit delay, while thioperamide (a mixed H<sub>3</sub>R/H<sub>4</sub>R antagonist), unlike the selective H<sub>3</sub>R antagonist M85, reduced leukocytes infiltration in ischemic mice.

In the gut, mast cells are often in close vicinity to visceral afferents that express Proteinase-Activated Receptor 2 (PAR<sub>2</sub>)<sup>30</sup>. We showed that PAR<sub>2</sub> activation has a protective role in post-ischemic reperfusion induced injury in the intestine: in fact, intraduodenal administration of the selective PAR<sub>2</sub> agonist SLIGRL-NH<sub>2</sub> reduced

mucosal damage and accelerate gastrointestinal transit in I/R animals. The inflammation caused by I/R might induce degranulation of mast cells with release of proteases, which activates PAR<sub>2</sub> directly and/or through activation of visceral afferents. The mechanism underlying the protective effects of PAR<sub>2</sub> activation appears to be mediated by mast cells degranulation and sensory afferents, because it was not observed in rats pretreated with cromolyn (a mast cell stabilizer) and chronic capsaicin (which ablates visceral afferents), with a mechanism that involves at least in part substance P release<sup>31</sup>.

### **1c. SYSTEMIC CONSEQUENCES OF INTESTINAL I/R**

Localized mesenteric ischemia/reperfusion event can trigger more severe systemic disorders associated to a high morbidity and a mortality rate which ranges from 60% to 100%<sup>3</sup>. In fact, damage to the microcirculation leads, locally, to irreversible intestinal necrosis, and disseminated intravascular coagulation (DIC) or systemic inflammatory syndrome (SIRS) in the whole body or in remote organs.

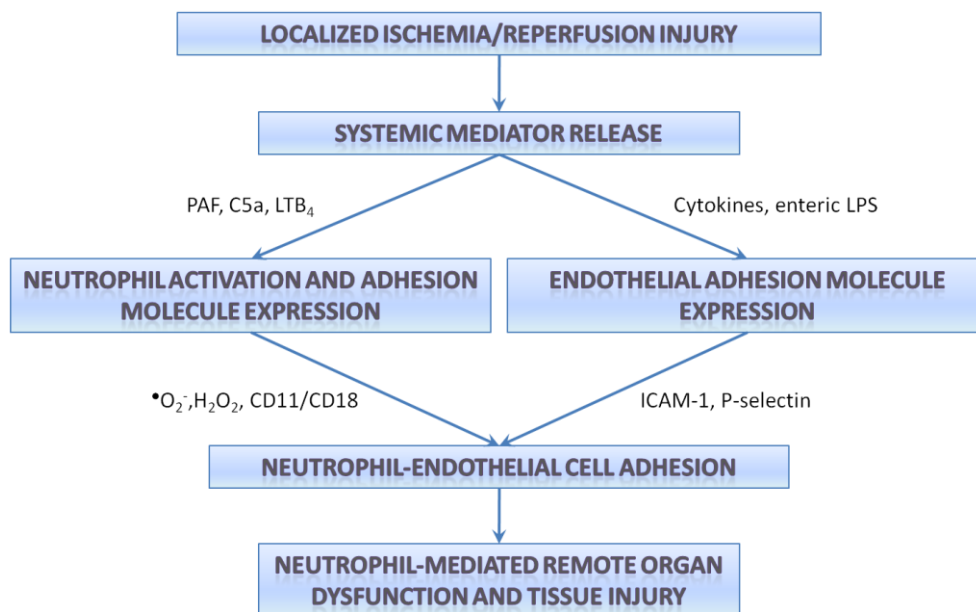
During intestinal I/R, activated neutrophils infiltrate through endothelial cells, increasing vascular permeability and causing sub-mucosal and mucosal damage. This increase in permeability often lead to translocation of enteric bacteria and bacterial products (e.g. LPS) from the gastrointestinal tract to extra-intestinal sites like lymph nodes, spleen and liver. Subsequently bacteria are able to disseminate to the entire body, causing sepsis, shock or even multiple organ failure (MOF) and death. MOF is characterized by dysfunction of immune system and activation of the coagulation cascade resulting in thrombosis, disseminated intravascular coagulation and immune-failure.

After I/R insult, production and release of pro-inflammatory cytokines and radical substances, such as superoxide anion or hydrogen peroxide, can lead to severe systemic consequences. In fact, mediators released into circulation during reperfusion are able to activate endothelial cells in any distant organs and to enhance expression of adhesion molecules and interaction between neutrophils and endothelial cells in different vascular beds. The most frequent damage is pulmonary edema associated with accumulation of neutrophils-rich alveolar fluids which can lead to respiratory failure and acute respiratory distress syndrome (ARDS)<sup>32</sup>. Other remote organ injuries often following



intestinal I/R events include systemic inflammatory response syndrome (SIRS), cardiac dysfunction, renal failure and hepatic injury.

Another systemic consequence of intestinal I/R can be the insurgence of alteration in absorptive functions of the small intestine, which can lead to deficient absorption of nutrients, often associated with an increased mortality<sup>33</sup>.



**Figure 5:** Systemic consequences of local Ischemia/Reperfusion injury. Schematic illustration of the mechanism involved in the development of remote organ dysfunction. (modified from *Carden D.L., Granger D.N., J Pathol 2000*)

## **1d. THERAPEUTIC APPROACHES TO I/R INJURY**

Several potential therapeutic approaches have been proposed for treatment of ischemia/reperfusion injury, and a variety of different drugs and therapeutic strategies have shown protective effects in experimental animal model of intestinal I/R injury. Among the strategies which have been successfully used, the most noteworthy are:

- ✓ Ischemic preconditioning
- ✓ Antioxidants administration
- ✓ Anti-complement therapy
- ✓ Anti-leukocyte therapy
- ✓ Hypothermia
- ✓ iNOS inhibition

Ischemic preconditioning (IPC) consists in exposure of the intestinal tissue to one or more brief period of ischemia, which is demonstrated to protect from the damaging effects of a subsequent prolonged ischemic event. This protective effect of IPC was first described on canine heart by Murry and colleagues in 1986<sup>34</sup>, and subsequently demonstrated also in intestinal tissue by Hotter in 1996<sup>35</sup>. IPC is able to prevent leukocyte activation and adhesion, capillary plugging and extra-vascular leaking of proteins, preserving, in this way, endothelial functions of arteries, capillaries and venules and diminishing morphological lesions in the entire intestinal tissue.

IPC protective effects can be distinguished in two different phases: (1) *early* or classic preconditioning and (2) *late* preconditioning. Early IPC occurs within minutes after the initial transient ischemic insult and the beneficial effect last at least 2-3 hours. This early phase involves direct modulation of specific cell functions (e.g. stimulation

of phospholipase C, activation of protein kinase C) and is protein synthesis-independent. The acute protection afforded by early IPC is lost if the interval of time that elapses between the transient and the prolonged ischemia exceeds the 2-3 hours. However, a delayed protective response appears again if the prolonged ischemia occurs 12-24 hours after the initial vascular occlusion and lasts for about 3-4 days. This late preconditioning phase involves the activation of multiple stress response genes and requires de novo synthesis of various proteins, including antioxidant enzymes, NO synthase and heat shock proteins.

Several hypothesis have been proposed to explain these events, but the exact mechanism through which IPC exerts its protective role is still unclear. In the gut IPC is associated to an increased mucosal activity of the antioxidant enzymes superoxide-dismutase (SOD), catalase and glutathione peroxidase (GSH)<sup>36</sup> and to augmented NO production. Thus, IPC appears to enhance target tissue's antioxidant status, improving the innate resistance to a second ischemic insult. Evidences showed that in the small intestine, IPC seems to prevent post-ischemic leukocyte adhesion by maintaining the bioavailability of nitric oxide (a potent endogenous anti-adhesive agent) and preventing the expression of adhesive molecules by endothelial cells (P-selectin)<sup>37</sup>. In addition, IPC is able to decrease the systemic translocation of bacteria from the intestine<sup>38</sup>.

Since opioid peptide have been detected in the gut and their receptors were also found to be widely expressed in the small intestine, the role of endogenous opioid peptide in the protective effect of IPC on intestinal ischemic injury has been investigated. Studies on experimental model of intestinal I/R in rats showed that pretreatment with morphine, an opioid receptor agonist, could produce a preconditioning-like protection against damage caused by a subsequent I/R; whereas

pretreatment with the opioid receptor antagonist naloxone significantly blocked both IPC- and morphine-induced protection. Moreover, evidence that the release of the endogenous opioid peptide Leu-enkephalin was markedly increased during IPC, suggests that endogenous opioid peptide may mediate the protective effects of intestinal IPC and may also be an endogenous protective substance in the rat small intestine<sup>39</sup>.

Antioxidants are molecules provided of free radical scavenging properties, which are helpful in reducing damage induced by the oxidative burst occurring during I/R. A variety of molecules have been successfully used in experimental model of ischemia, including xantine-oxidase inhibitor (e.g. allopurinol), Superoxide Dismutase, iron chelator, N-acetyl cysteine, ethanol, ascorbic acid, tocopherol, pentoxifylline, captopril and verapamil<sup>1</sup>.

Anti-complement therapy consists in complement inhibition or depletion through administration of complement (C5a) receptor antagonist or anti-murine C5 monoclonal antibody. This therapeutic approach has been shown to protect against intestinal I/R injury in rodents, attenuating intestinal mucosal injury and decreasing neutrophil infiltration<sup>22, 40</sup>.

Anti-leukocytes therapy aims to prevent or attenuate the intestinal I/R damage mediated by immune cells. Three different strategies have been tested in this field: (1) *inhibition of leukocyte activation*, by administration of antagonists of pro-inflammatory mediators which attract and activate leukocytes during I/R (e.g. TNF- $\alpha$  antagonists<sup>14</sup>, LT-B<sub>4</sub> antagonist<sup>41</sup>); (2) *inhibition of leukocyte adhesion molecule synthesis* through inhibition of nuclear transcription factors regulating genes codifying for adhesion proteins (e.g. NF- $\kappa$ B inhibitor<sup>12</sup>); (3) *limitation of leukocyte-endothelial adhesion* using

monoclonal antibody directed against adhesion molecules and preventing PMNs rolling and adherence on endothelial vascular cells<sup>42</sup>.

Intestinal hypothermia, experimentally induced in rats by keeping tissue temperature around 15-20°C, is able to protect the intestinal mucosa from mesenteric I/R injury acting, probably, with a multi-factorial mechanism. Indeed, hypothermia reduces consumption of oxygen and ATP, neutrophil infiltration, lipidic peroxidation and production of pro-inflammatory cytokines. In addition, hypothermia also prevents NF- $\kappa$ B activation and modulates expression of the oxidative stress protein iNOS in response to mesenteric I/R<sup>43</sup>.

As previously described, NO plays a double role in pathophysiology of intestinal I/R. Supplementation of NO, achieved through treatment with NO precursor or donors, has been shown to ameliorate local and systemic I/R injury when administered before the ischemic event. On the contrary, the reduction of NO tissue levels, gained through the selective inhibition of the iNOS isoform during the post-ischemic period, protects intestine from both motor disturbance and inflammatory response induced by mesenteric I/R in mice<sup>44</sup>.

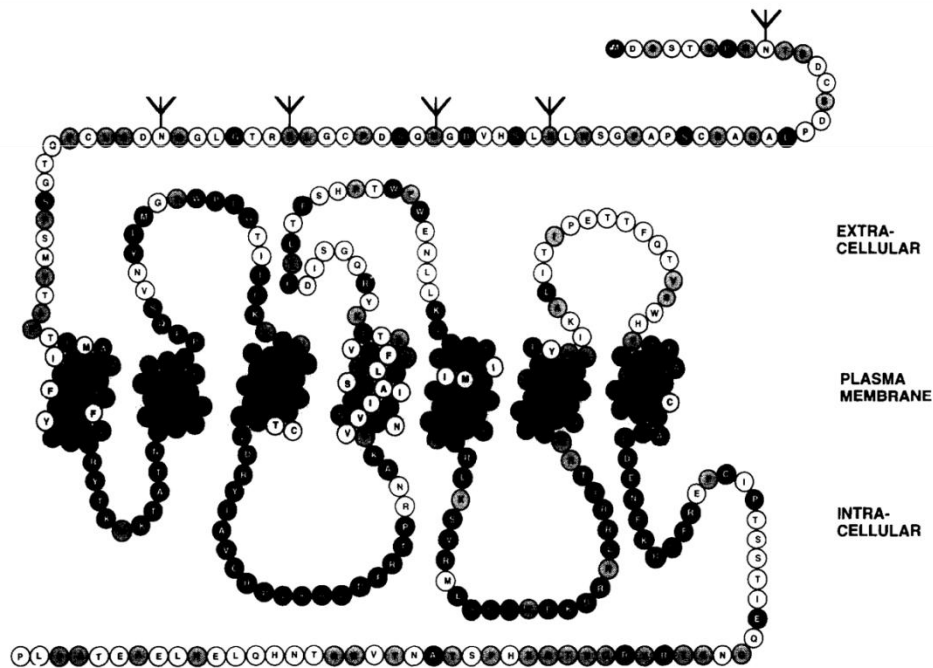
## 2. $\mu$ OPIOID RECEPTOR

### 2a. OPIOID RECEPTORS

Opiate derivatives like morphine and other alkaloids have been used in medicine for their analgesic properties from ancient times. The word *opium* itself is derived from the Greek name for juice, the drug being obtained from the juice of the poppy *Papaver Somniferum*. Opium contains more than 20 alkaloids (e.g. morphine, codeine, papaverine) characterized by different pharmacological properties.

The existence of opioid receptors, through which opiates exert their action, was first proposed in 1954 by Beckett and Casy<sup>45</sup> based on their studies of structure-activity relationships for antinociceptive activity in a series of synthetic opiates<sup>45</sup>. Portoghesi and colleagues first suggested in 1965 the existence of more than one opioid receptor type or the possible occurrence of multiple modes of interaction of ligands with opioid receptors<sup>46</sup>. In 1975 Hughes and Kosterlitz isolated from cerebral tissue two pentapeptides sharing a similar pharmacological profile of morphine thus strengthening the idea of the existence of specific receptor targets. The first definitive evidence that these opioid receptors did not form an homogeneous population was presented by Martin and colleagues in 1976<sup>47</sup>. They proposed that there were three types of opioid receptors based on the pharmacological actions of different opiates in chronic spinal dogs, and named them  $\mu$  ('mu' for morphine),  $\kappa$  ('kappa' for ketocyclazocine),  $\sigma$  ('sigma' for SKF 10047 or N-allylnormetazocine). Few years after, Lord and colleagues identified a high affinity receptor for opioid receptor ligands in mouse vas deferens and named it  $\delta$  ('delta' for vas deferens)<sup>48</sup>.

The amino acid sequences of all these receptors have been cloned and the recombinant receptors shown to have binding and functional characteristics consistent with their endogenous counterparts<sup>49</sup>. There is about 60% of homology between these receptors and each one has seven trans-membrane domains characteristic of the G-protein coupled receptor (GPCR) family.



**Figure 6:** Amino acid sequence identity among the three cloned opioid receptor. Amino acid residues of  $\mu$ OR conserved in both  $\delta$ - and  $\kappa$ -receptor, in either  $\delta$ - or  $\kappa$ -receptor and in neither  $\delta$ - nor  $\kappa$ -receptor are shown by black, gray and white circles, respectively. Branched structures show the potential N-linked glycosylation sites. [Minami M. and Satoh M. Neurosci Res 1995]

Opioid receptors are associated to  $G_{\alpha i/o}$  and their activation inhibits adenylyl cyclase and  $Ca^{2+}$  channels, while opens  $K^+$  channels, generating changes in membrane polarization and inhibition of neurotransmitters release.

Once presence of specific receptors for opiate alkaloid and related synthetic drugs was confirmed, the investigation for endogenous ligands for these receptors began and led to discovery of opioid peptides: Leu-enkephalin and Met-enkephalins<sup>50, 51</sup>,  $\beta$ -endorphin<sup>51, 52</sup>, and dynorphins<sup>53</sup>. Opioid receptors have specific pharmacological

profile and a certain degree of selectivity for different endogenous ligands. For instance, endorphins bind to  $\mu$ - and  $\delta$ -receptors with similar affinity, whereas dynorphine displays some selectivity for  $\kappa$ -receptor and enkephalins are the preferred ligands for  $\delta$ -OR, but also have remarkable affinity for  $\mu$ -OR<sup>54</sup>. The recently discovered opioid peptides, endomorphine-1 and endomorphine-2, have several thousand-fold preference for  $\mu$ OR over  $\delta$ - and  $\kappa$ -receptor.

**Table 1:** Endogenous opioid peptides selectivity among opioid receptors and their amino acid sequences.

Opioid peptide	Receptors	Amino acids sequence
Met-enkephalin	$\delta > \mu$	Tyr-Gly-Gly-Phe-Met
Leu-enkephalin	$\delta > \mu$	Tyr-Gly-Gly-Phe-Leu
$\beta$ -endorphin	$\mu > \delta$	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
Dynorphin A (1-17)	$\kappa$	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
Endomorphin-1	$\mu$	Tyr-Pro-Trp-Phe-NH <sub>2</sub>
Endomorphin-2	$\mu$	Tyr-Pro-Phe-Phe-NH <sub>2</sub>

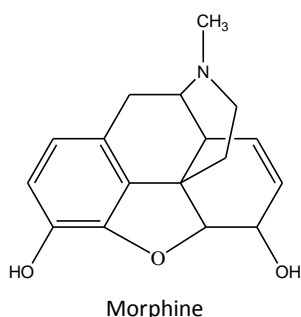
Endogenous opioids are generated from three different genes, the pro-opiomelanocortin (POMC), pro-enkephalin (PENK) and pro-Dynorphin genes, which give rise to several biologically active peptides, including  $\beta$ -endorphin, enkephalin and dynorphin, whereas, the genes producing endomorphins have not been yet identified. These genes and their products have a broad distribution in the central and peripheral nervous system as well as into other organs and tissues, including the gastrointestinal tract<sup>55</sup>.



## 2b. $\mu$ OPIOID RECEPTOR

The  $\mu$  opioid receptor subtype was originally defined and characterized pharmacologically on the basis of its high affinity for, and sensitivity to, morphine<sup>56</sup>. The endogenous opioids  $\beta$ -endorphin, [Met<sup>5</sup>]-enkephalin, [Leu<sup>5</sup>]-enkephalin, extended forms of [Met<sup>5</sup>]-enkephalin and truncated forms of dynorphin (e.g. dynorphin-(1-9) and shorter dynorphin peptides), have affinities for  $\mu$  receptors which are consistent with a possible role for each of these peptides as natural ligands for this receptor type, although these endogenous mediators are not selective for  $\mu$  receptors. Recently discovered, endomorphin-1 and -2 appear to mediate their effects exclusively through the  $\mu$  opioid receptor, although no gene, precursor protein, or other mechanism for their endogenous synthesis has been identified.

Opiates used clinically preferentially activated  $\mu$ OR even though their ligand selectivity profiles are impaired when they accumulate at high concentration, in which conditions they can interact with other opioid receptor subtypes<sup>57</sup>.  $\mu$ OR exogenous ligands can be subdivided in two groups based on their molecular structure: peptides (obtained modifying the endogenous opioid amino acid sequences) or non-peptides (gained modeling the opium alkaloids structures).



Among the selective exogenous agonists for  $\mu$  opioid receptor, most known are morphine, fentanyl, sufentanyl, methadone, DAMGO and dermorphin; while selective antagonist are CTAP, CTOP, naloxonazine, MET-CAMO [5-beta-methyl-14-beta- (p-nitrocinnamoylamino) -7,8-dihydro morphinone] and its corresponding N-cyclopropylmethyl analog (N-CPM-MET-CAMO).

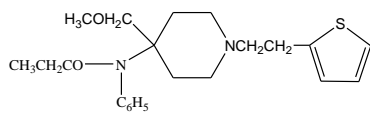
## μ opioid receptor AGONIST

### Peptides

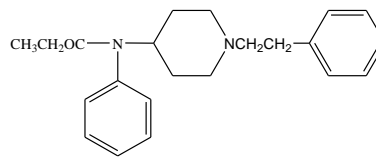
DAMGO	Tyr-D-Ala-Gly-MePhe-Gly-ol
FK 33-824	Tyr-D-Ala-Gly-MePhe-Met(O)-ol
PLO17	Tyr-Pro-τ-MePhe-D-Pro-NH <sub>2</sub>
Dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH <sub>2</sub>
Morphiceptin	Tyr-Pro-Phe-Pro-NH <sub>2</sub>

### Non-peptides

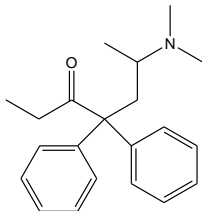
#### Sufentanyl



#### Fentanyl



#### Methadone



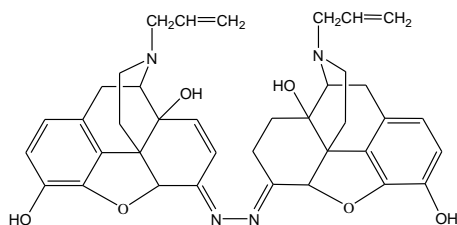
## μ opioid receptor ANTAGONIST

### Peptides

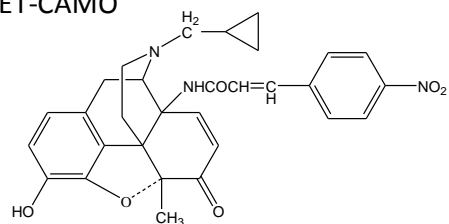
CTOP	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH <sub>2</sub>
CTP	D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH <sub>2</sub>
CTAP	D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH <sub>2</sub>
TCTOP	D-Tio-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH <sub>2</sub>

### Non-peptides

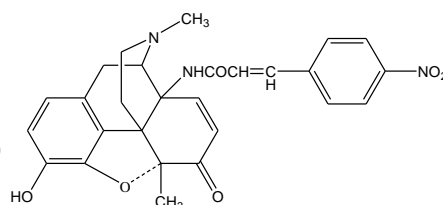
#### Naloxonazine



#### N-CPM-MET-CAMO

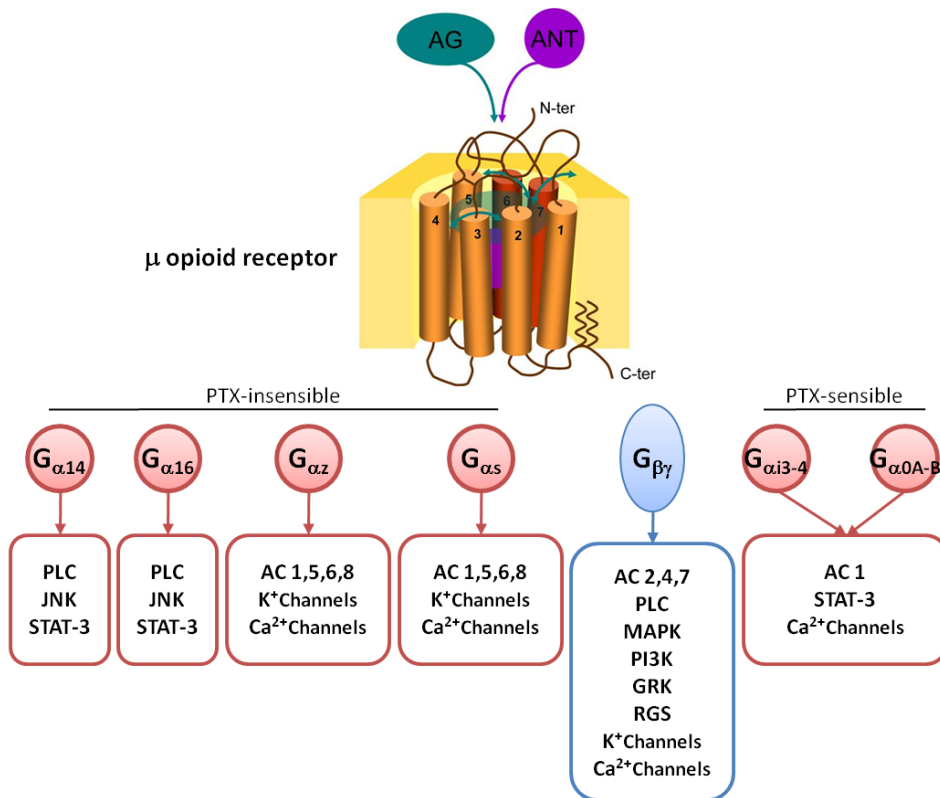


#### MET-CAMO



$\mu$ ORs are members of seven transmembrane G-protein-coupled receptors super family and are able to activate different signal transduction systems. These receptors can interact with five different  $G_{i/0}$  proteins ( $G_{i/1-2-3}$ ,  $G_{0/A-B}$ ) and are functionally coupled to several effectors pathways, including inhibition of adenylyl cyclase and cAMP formation, increase of  $K^+$  currents, inhibition of  $Ca^{2+}$  channels, modulation of inositol triphosphate ( $IP_3$ ) turnover and activation of mitogen-activated protein kinases (MAPK)<sup>58</sup>.  $\mu$ ORs can also interact with pertussis toxin-insensitive G proteins like  $G_z$  or  $G_{14-16}$ , which respectively inhibit cAMP production and activate phospholipase C (PLC). Moreover, the  $G_{\beta\gamma}$  complex is able to activate other various signaling pathways itself, generating different responses sometimes going in opposite direction compared to  $G_{i/0}$  effects. However, the  $G_{\beta\gamma}$ -correlated transduction system seems to be consequence of chronic activation of  $\mu$ OR by agonists, while  $G_{i/0}$  response results prevalently after acute stimuli. Coupling of the  $\mu$ OR to these effector systems attenuates neuronal activity by inhibiting neurotransmitters release and changing neuronal excitability by means of pre- and post-synaptic mechanisms.

After ligand-receptor interaction, the  $\mu$ OR undergoes adaptation such as desensitization, down-regulation and re-sensitization in response to agonist treatment. These events regulate cellular responsiveness to receptor activation and result from receptor mediated processes including phosphorylation, endocytosis, intracellular sorting and recycling<sup>59</sup>. Indeed, immuno-histochemical studies showed that  $\mu$ OR undergoes rapid ligand-induced receptor internalization in both the soma and neuronal processes which persists for 4-6 hours; and that after appropriate intracellular sorting, requiring endosomal acidification,  $\mu$ OR recycles to the cell surface in about 6 hours<sup>54</sup>.



**Figure 7:** Different transduction systems activated by  $\mu$ OR. This receptor can be coupled with different  $G_{\alpha}$  subunits, and also  $G_{\beta\gamma}$  complex can stimulate various signal pathways. AC = adeny cyclase, STAT-3 = Signal Transducer and Activator of Transcription-3, PLC = Phospholypase C, MAPK = Mitogen Activated Protein Kinase, PI3K = Phosphoinositide 3-Kinase, GRK = G-Protein-coupled Protein Kinase, JNK = c-Jun N-terminal Kinase, RGS = Regulator of G-Protein Signaling Protein. [modified from *Clementi F. UTET, 2004*<sup>60</sup>].

Pharmacological studies performed with selective agonists and antagonists, suggested the presence of multiple  $\mu$  opioid receptors subtypes referred to as  $\mu_1$  and  $\mu_2$  and  $\mu_3$ <sup>61</sup>:

✓  $\mu_1$ -ORs are expressed in the brain and are characterized by their high affinity for naloxonazine and several opioid ligands (including morphine and DALDE).  $\mu_1$ -ORs and are involved in various opioid effects such as supraspinal analgesia, prolactin release, decrease in acetylcholine turnover and induction of catalepsy<sup>62</sup>.

✓  $\mu_2$ -ORs are expressed in the brain as well as in the gastrointestinal and respiratory tract; they have lower affinity for morphine and DALDE and appear to be

involved in respiratory depression, decreased dopamine turnover and delayed GI transit induced by opiates<sup>54</sup>.

✓  $\mu_3$ -ORs are localized on human peripheral monocytes and in invertebrate immunocytes, they have been characterized by binding assays as opiate alkaloid selective (e.g. morphine) and opioid peptide (e.g. Met-, Leu-enkephalin) insensitive.  $\mu_3$ -ORs are coupled to constitutive nitric oxide synthase-derived NO release and are involved with neural, immune, vascular and gut processes<sup>63</sup>.

**Table 2:** Features of human  $\mu$  opioid receptors subtypes.

	$\mu_1$ OR	$\mu_2$ OR	$\mu_3$ OR
<b>Tissue distribution</b>	Brain	Brain, Spinal cord Gastrointestinal tract Respiratory tract	Monocots, granulocytes, vascular endothelial cells, nervous tissue
<b>Signal transduction</b>	Decrease: Ca <sup>2+</sup> and K <sup>+</sup> channels cAMP	Decrease: Ca <sup>2+</sup> and K <sup>+</sup> channels cAMP	Increase: Ca <sup>2+</sup> and constitutive NO release

## **Localization and functions**

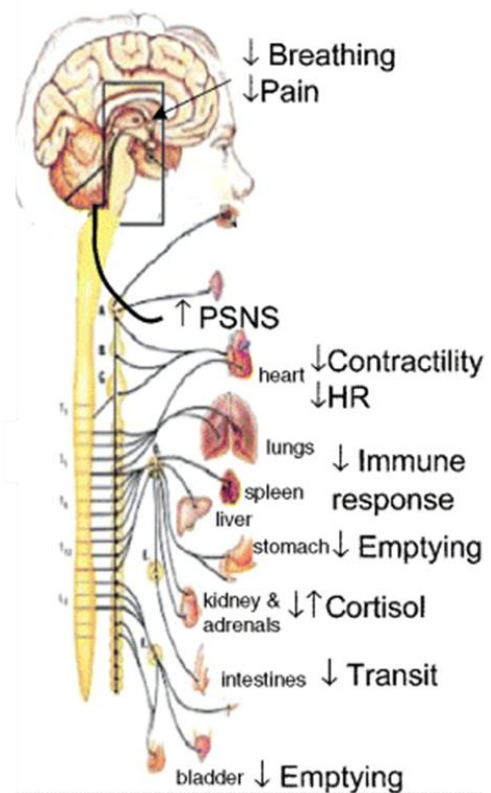
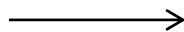
*In situ* hybridization studies using highly selective probes for mRNA of the various opioid receptor subtypes have revealed that  $\mu$ ORs are distributed throughout the neuraxis. Into the Central Nervous System (CNS) the highest  $\mu$  receptor densities are found in periaqueductal gray substance, spinal trigeminal nucleus, cuneate and gracile nuclei, and thalamus regions of the brain involved in pain perception<sup>64</sup>.  $\mu$ ORs mRNA is present also in nuclei involved in control of respiration (solitary tract, ambiguous and parabrachial), in areas processing memory, emotional reactions and behavior (amygdale, neocortex, nucleus accumbens), and in neurons of the area postrema able to induce nausea and vomiting<sup>65</sup>. The  $\mu$ ORs are also present in the superficial layers of the dorsal horn of spinal cord (dorsal root ganglia) and peripheral nerve terminals<sup>66</sup>.

Activation of  $\mu$  opioid receptor by endogenous opioid peptides and opiate drugs modulate a variety of physiological functions and biological processes, including analgesia, stress response, immunity, motor activity and autonomic functions acting centrally as well as peripherally<sup>67</sup>.

✓ Stimulation of central  $\mu$ OR induces analgesia, by reducing both physical and psychic pain, produces drowsiness, apathy and sedation and also acts like a “mood controller” triggering euphoria and tranquility and, at the same time, minimizing anxiety and anguish. Moreover,  $\mu$ OR activation within the CNS generates severe respiratory depression, by reducing responsiveness of brainstem respiratory centre to carbon dioxide; miosis, body temperature adaptation and is able to control feeding stimuli, inducing anorexia, nausea and vomiting acting on chemoreceptor trigger zone for emesis, in the area postrema of the medulla.

✓ Consequences of peripheral activation of  $\mu$ OR include inhibition of gastrointestinal transit and motility, due to increase of smooth muscle basal tone, enhance of non-propulsive contractions and marked decrease of propulsive contractions.  $\mu$ OR are able to modulate gastrointestinal functionality since they trigger a decrease in gastric, biliary, pancreatic and intestinal secretions. Alteration of various hormones secretion is another consequence of  $\mu$ OR activation: increased release of prolactin (PRL) and growth hormone (GH), inhibition of luteinizing hormone (LH) release and urinary retention correlated to an increased release of antidiuretic hormone (ADH) are detected. Furthermore,  $\mu$ OR activation produces peripheral vasodilatation, reduces peripheral resistance and heart rate and inhibits baroreceptor reflex causing orthostatic hypotension. Morphine and some opioids also release histamine from mast cells causing a rapid decrease of blood pressure and emerging of itch.

$\mu$  opioid  
receptor  
activation



**Figure 8:** Endogenous opioids and pharmacologically administered opiate analogues bind and activate  $\mu$ ORs, which exert their effects in multiple central and peripheral target cells producing respiratory depression, analgesia, negative inotropic effect, immune-suppression, GI function alteration. [modified from Molina PE, *J Intern Med* 2006]

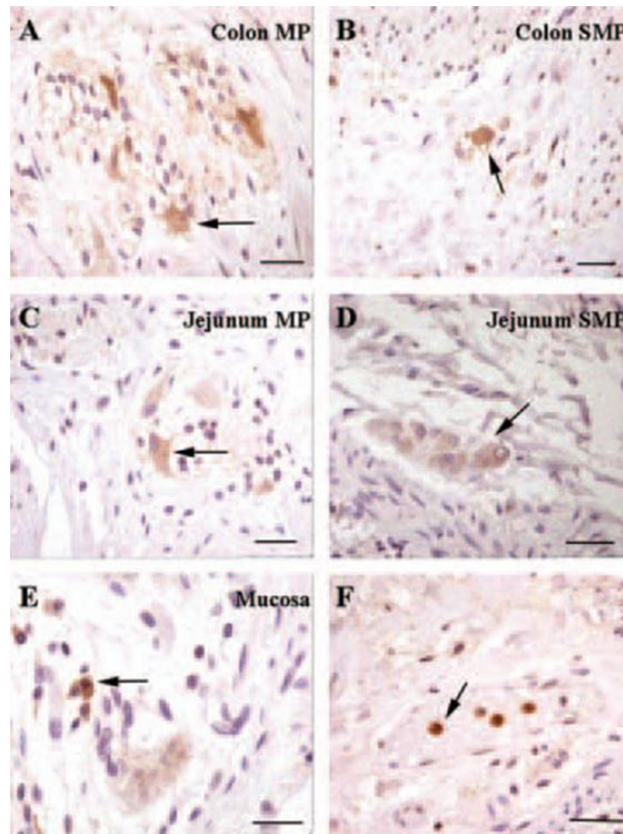
## 2c. $\mu$ OR IN THE GASTROINTESTINAL TRACT

The enteric nervous system (ENS) represents the largest collection of neurons outside the CNS. Enteric neurons originating from the myenteric and submucosal plexuses supply all layers of the gastrointestinal wall and exert essential roles in controlling bowel movement and transmucosal fluid exchange. Due to these features ENS is able to regulate every aspect of digestion processes<sup>68,69</sup>. These neurons are able to synthesize and release acetylcholine (ACh), substance P, nitric oxide (NO), adenosine triphosphate(ATP), vasoactive intestinal polypeptide (VIP), 5-hydroxy-tryptamine (5HT) and also opioid peptides (b-endorphin, enkephalin, endomorphin)<sup>70</sup>. Unlike other nervous system in the body, the ENS can work without central input from the brain and is often considered “the brain in the gut”.

$\mu$  opioid receptor has been localized in enteric neurons of various animal species and their relative distribution varies with gastrointestinal layer and region. In the rat  $\mu$ OR has been observed in neurons of both the submucosal and myenteric plexus and in fibers distributed to the muscle layers, mucosa, blood vessels, lymphatic nodes and to putative interstitial cells of Cajal in the myenteric and deep muscular plexus<sup>71</sup>. By contrast, in guinea-pig,  $\mu$ OR is localized predominantly to neurons of the myenteric plexus, which are more abundant in the small intestine, particularly the ileum, than in the stomach and proximal and to fibers distributed to the interconnecting strands and smooth muscle layer, where they form a dense network in the deep muscular plexus<sup>71</sup>. In human gut,  $\mu$ OR is present in specific regions of the small and large intestine: distribution and density are comparable in the human jejunum and colon.  $\mu$ OR immunoreactivity is localized in neuronal cell bodies in both submucosal and myenteric ganglia and to nerve



fibers in the myenteric plexus, but not to the mucosa.  $\mu$ OR are present on mononuclear cells in the lamina propria and on neutrophils in the microcirculation<sup>72</sup>.



**Figure 9:**  $\mu$ OR distribution in human gut specimens.  $\mu$ OR immunoreactivity is found in (A and C) myenteric neurons, (B and D) submucosal neurons, and (E and F) mucosal immune cells. Arrows point to  $\mu$ OR in neurons of the myenteric plexus of (A) the colon and (C) the jejunum, of the submucosal plexus of (B) the colon and (D) the jejunum, and in (E and F) immune cells. MP, myenteric plexus; SMP, submucosal plexus. Calibration bars: 50  $\mu$ m. [Sternini C. et al. *Neurogastroenterol Motil* 2004]

The  $\mu$ OR immunoreactivity distribution in the gastrointestinal tract closely matches the distribution of the opioid peptides enkephalins<sup>73</sup>, and these molecules are likely to be the endogenous opioids that primarily activate neuronal  $\mu$ OR in the gut, even though they are not selective for this opioid receptor type<sup>55</sup>.

$\mu$ OR expressing myenteric neurons comprise functionally distinct types of neurons: including cholinergic and tachykinergic excitatory motoneurons ascending to the

muscle<sup>54</sup> and ascending interneurons<sup>74</sup>. In addition,  $\mu$ OR expressing enteric neurons comprise a large proportion of descending neurons and cholinergic descending interneurons.

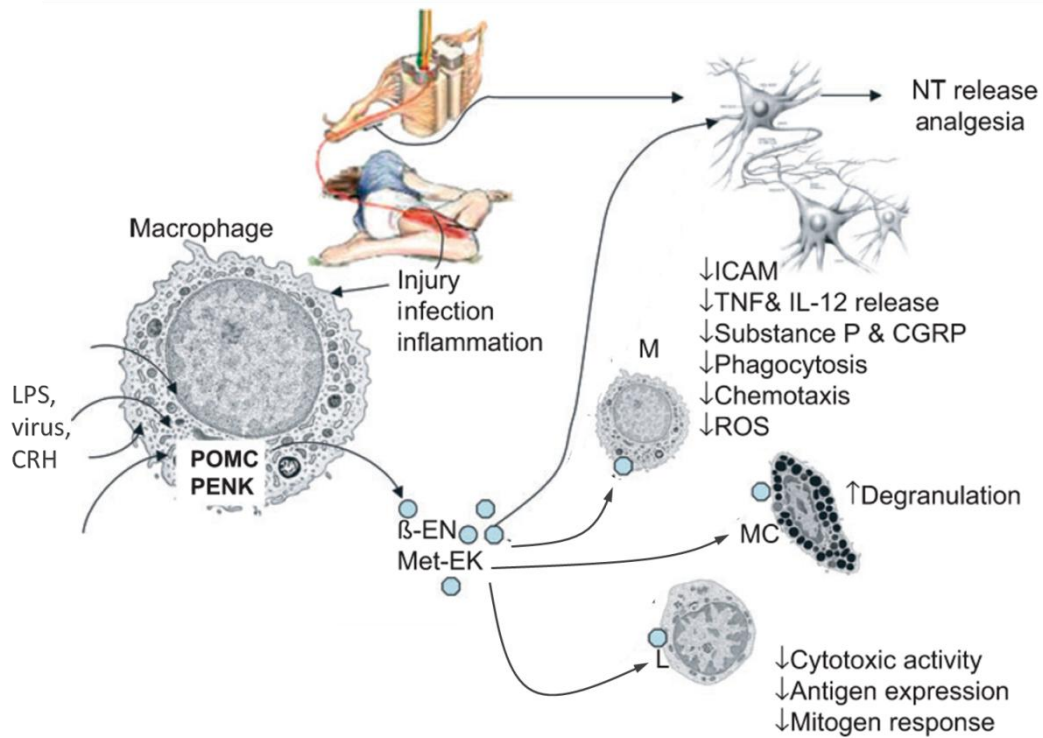
Due to this widespread distribution activation by agonist of  $\mu$ OR receptor can interrupt both excitatory and inhibitory neural inputs to GI muscles<sup>75</sup>. The consequent motor effects are complex because, depending on whether interruption of excitatory or inhibitory neural pathways prevails, muscle relaxation or spasm will be observed. As a result,  $\mu$ OR agonists inhibit gastric emptying, increase pyloric muscle tone, induce pyloric and duodenal-jejunal phasic pressure activity, delay transit through the small and large intestine, and elevate the resting anal sphincter pressure<sup>75</sup>. The halt in propulsive motility combines with inhibition of gastrointestinal ion and fluid transport. Through prolonged contact of the intestinal contents with the mucosa and interruption of pro-secretory enteric reflexes,  $\mu$ OR activation attenuate the secretion of electrolytes and water and facilitate the net absorption of fluid, resulting in a pronounced constipation<sup>76</sup>.

## 2d. $\mu$ OR AND IMMUNE RESPONSE

Opioid receptor activation occurs as a natural response to injury resulting from increased release of opioid peptides in response to injury, infection, trauma and surgery. Although the most frequently associated effect of opioid is analgesia, several lines of evidence suggest that endogenous and pharmacological activation of opioid receptors affects immune function and can regulate immune response<sup>77</sup>.

It is proved that immune cells recruited in inflamed tissue, such as T and B lymphocytes, granulocytes, monocytes and macrophages, contain all subtype of opioid peptides (endorphin, enkephalin, dynorphin and endomorphin). Pro-opiomelanocortin (POMC) -related opioid peptides have been found in leukocytes of many vertebrates and invertebrates<sup>78</sup>, while pro-enkephalin (PENK)-related have been detected in human and rodent T and B cells, strongly indicating that opioid peptides derive from the same precursors both in nervous and immune system<sup>79</sup>. Furthermore,  $\beta$ -endorphin is present in secretory granules of macrophages and leukocytes, arranged at the cell periphery, ready for exocytosis<sup>80</sup>. Indeed, leukocytes are capable of directly processing and releasing POMC- and PENK-derived peptides when subject to various stressful stimuli or in response to releasing agents such as viruses, corticotropin-releasing hormone (CRH), cytokines (e.g. IL-1 $\beta$ ), chemokines (e.g. CXCL1, CXCL2), and catecholamines (e.g. noradrenalin).

Opiate mediated immune effects result from either direct interaction with  $\mu$ OR on cells of the immune system<sup>81</sup> or indirectly, through the activation of opioid receptors within the central nervous system and the resulting modulation of hypothalamic-pituitary-adrenal axis and the sympathetic nervous system<sup>82</sup>



**Figure 10:** Opioid mediated immunomodulation and immunosuppression. L, lymphocytes, M, macrophages; MC, mast cells; ICAM; intercellular adhesion molecule; ROS, reactive oxygen species; β-EN, β-endorphin; Met-EK, Met-enkephalin. [modified from *Molina PE, J Intern Med 2006*]

The locally produced opioid peptides can bind to  $\mu$ OR located on macrophages, lymphocytes and mast cells, and exert various immunomodulatory effects including reduced expression and release of adhesion molecules, tumor necrosis factor (TNF), substance P and calcitonin gene-related peptide (CGRP)<sup>67</sup>. Reports from in vivo and in vitro studies indicate that  $\mu$ OR stimulation exerts suppression of multiple components of the host immune defense response including natural killer cell activity<sup>83</sup>, neutrophil complement and immunoglobulin receptor expression<sup>84</sup>, chemokine-induced chemotaxis<sup>85</sup> and phagocytosis<sup>86</sup>. Opioid peptide can also produce immunosuppressive effects on T cells including inhibition of cytotoxic T-cell activity, modulation of T-cell antigen expression, and depression of responses to T-cell mitogens.

## **μOR and inflammatory response**

Besides the classical therapeutic properties of opioid drugs, the demonstration of opioid peptides and μ opioid receptors expression by cells involved in the inflammatory reaction has led to a new investigation focused on the roles of μOR ligands in the regulation and modulation of tissue inflammatory responses<sup>87, 88</sup>.

Inflammation of peripheral tissues leads to increased synthesis and axonal transport of opioid receptors in dorsal root ganglion neurons, resulting in their up-regulation in the peripheral nerve terminals<sup>89</sup>. Furthermore, opioid peptides, present in many leukocyte subpopulation including lymphocytes, monocytes and granulocytes in the peripheral blood, in inflamed and non-inflamed lymph node and also immune cells which extravasate in the site of experimentally induced or clinical inflammation. In early inflammation granulocytes are the major source of opioid peptide production; later in the inflammatory process, monocytes and macrophages are the predominant supply of these peptides<sup>90</sup>.

Recent evidence suggests that μOR could play a pivotal role in regulating intestinal inflammation. It is proved that intestinal inflammation experimentally induced by intragastric administration of croton oil in mice, enhanced the potency of μOR agonists in inhibiting gastrointestinal transit and secretion<sup>91, 92</sup>. Moreover, gut inflammation significantly increased the levels of μOR mRNA and receptor-protein located in the myenteric plexus suggesting that the enhanced effects of opioid observed in functional studies could be explained by an increase in the local synthesis and expression of the μOR<sup>93</sup>. Furthermore, in transperietal ileal and colonic samples of human patients suffering of inflammatory bowel diseases (IBD), compared to healthy intestine, a 6-30-

fold increased expression of  $\mu$ OR was observed<sup>94</sup>; supporting the hypothesis that up-regulation of  $\mu$ OR may operate a beneficial effect on abnormally accelerated intestinal transit and on the severity and duration of the inflammatory process.

Philippe and colleagues showed that subcutaneous administration of selective peripheral MOR agonists significantly reduces inflammation in two experimental models of colitis, and this effect was abolished by concomitant administration of an opioid antagonist<sup>95</sup>. Evidence of a genetic role for  $\mu$ OR in the control of gut inflammation was provided by showing that  $\mu$ OR-deficient mice were highly susceptible to colon inflammation. The anti-inflammatory effects of  $\mu$ OR in the colon appear to be mediated through the regulation of cytokine production and T cell proliferation<sup>95</sup>.

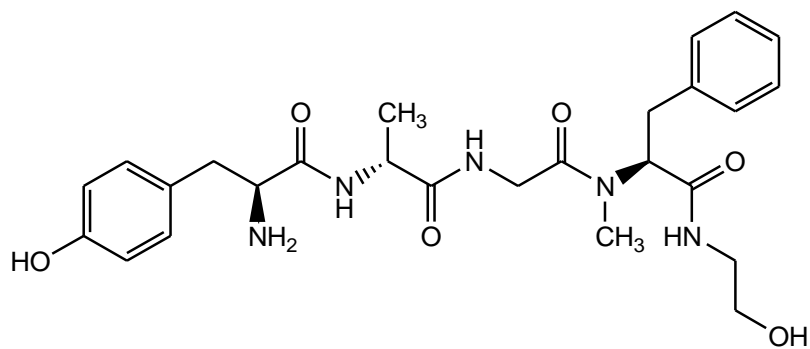
Taken together, these evidences support the hypothesis that  $\mu$ OR plays a role in the control of gut inflammation and suggest that  $\mu$ OR selective agonist, able to act peripherally without central untoward side effects such as depression of breathing, clouding of consciousness, or addiction, might be new therapeutic molecules for the treatment of inflammatory bowel disease.

## **AIM OF THE STUDY**

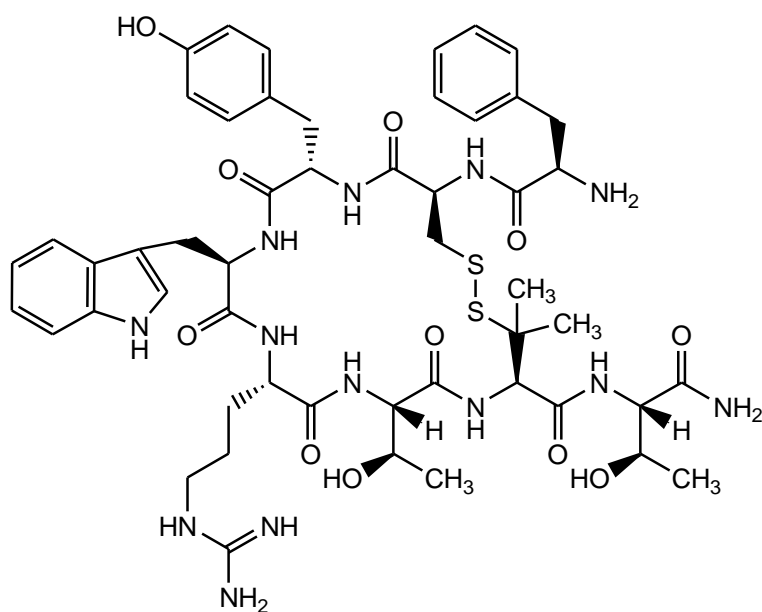
Considering recent evidences showing the involvement of  $\mu$  opioid receptor in controlling and regulating the intestinal inflammation, we hypothesized that  $\mu$ ORs stimulation might attenuate the inflammatory response as well as the mucosal injury induced by intestinal ischemia and reperfusion events. Aim of the study was to investigate the role of  $\mu$ OR stimulation in an experimental model of mild mesenteric ischemia developed in mice by reversible occlusion of the superior mesenteric artery (SMA) for 45 min followed by 5 hours of reperfusion<sup>26, 28, 31, 44, 96</sup>.

In order to test our hypothesis we evaluated the effect of exogenous administration of a selective  $\mu$ OR agonist (DAMGO) in presence or absence of a  $\mu$ OR antagonist (CTAP) on the intestinal injury induced by ischemia and reperfusion in mice. As index of local intestinal damage, we assessed changes in intestinal myeloperoxidase activity, mucosal histological integrity and ilea mRNA expression levels of interleukins (TNF- $\alpha$ , IL-1 $\beta$ , IL-10) involved in the inflammatory response. We evaluated the alterations of gastrointestinal motility, to test the integrity of gastrointestinal functionality and, finally, we checked if I/R insult induce any variation in  $\mu$ OR mRNA expression. We pondered results obtained for I/R mice treated with  $\mu$ OR agonist, antagonist or both, in comparison with I/R untreated or sham operated mice.





DAMGO



CTAP

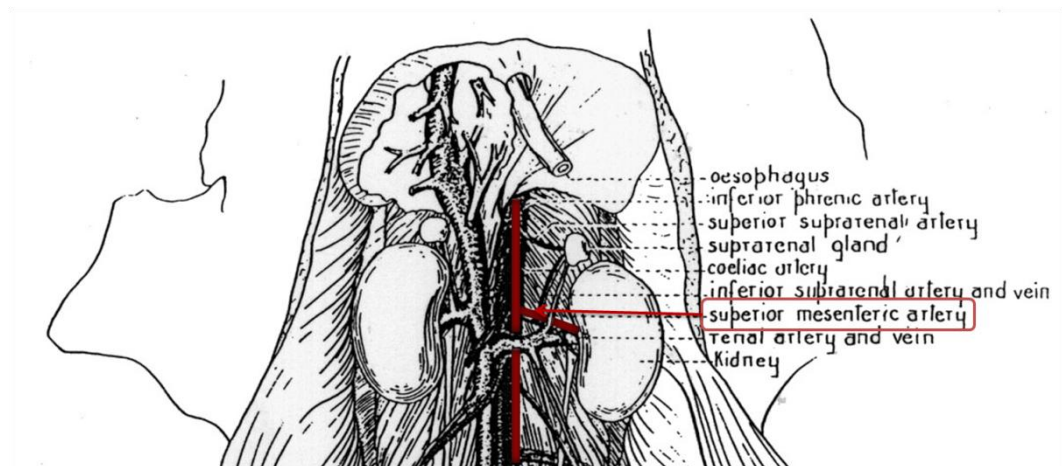
**Figure 11:** Molecular structure of the  $\mu$ OR selective agonist DAMGO ([D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin) and  $\mu$ OR selective antagonist CTAP (H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>).

## **MATERIALS AND METHODS**

Animal care and procedures were in accordance with the National Institute of Health recommendations for the humane use of animals. All experimental procedures were reviewed and approved by Animal Research Committee of the University of California, Los Angeles. Experiments were performed on female adult C57BL/6 mice (20-25g; Charles River Laboratories, Montreal, QC, Canada) that were housed under standard conditions and fasted, with free access to water, 12 hours before the experimental procedures.

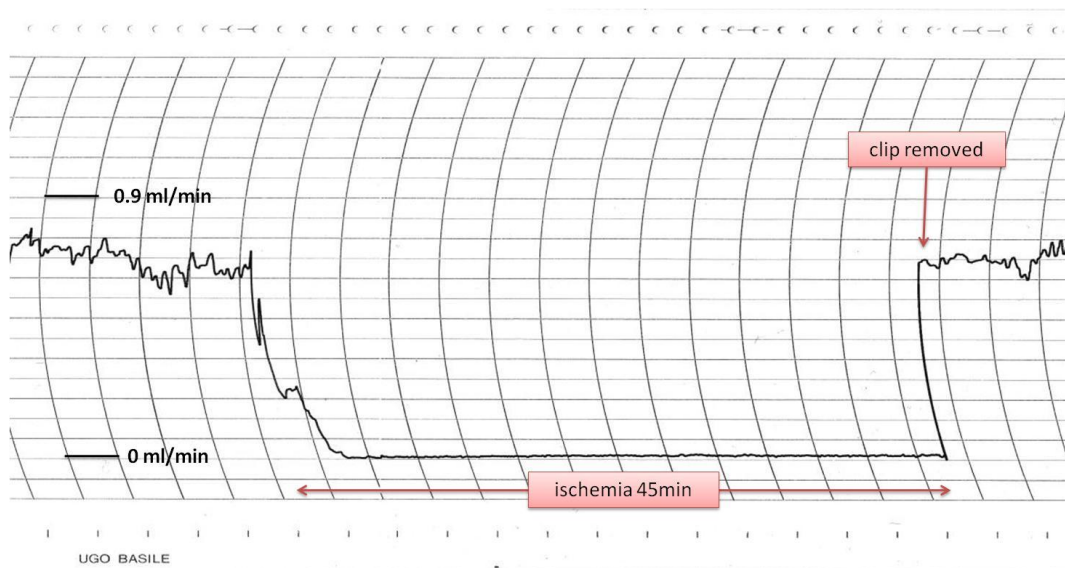
### **Ischemia/reperfusion and experimental design**

The study was performed using a model of mild mesenteric ischemia in mice previously set up by our group<sup>96</sup>. Animals were anaesthetized with intra-peritoneal injection of Nembutal® (pentobarbital) at the dosage of 50 mg/kg.



**Figure 12:** Anatomic localization of Superior Mesenteric Artery in mice.

Following abdominal laparotomy the small bowel was retracted to the left and the superior mesenteric artery (SMA) was identified and temporary occluded using a microvascular clip for 45 min. Then, by gently removing the clip, the reperfusion was allowed for a period of 5 hours and the abdominal cavity was closed by a two-layer suture. Monitoring of the bloodstream showed that the SMA occlusion generate a drop in arterial flow, which was completely reversed by removing the clips.



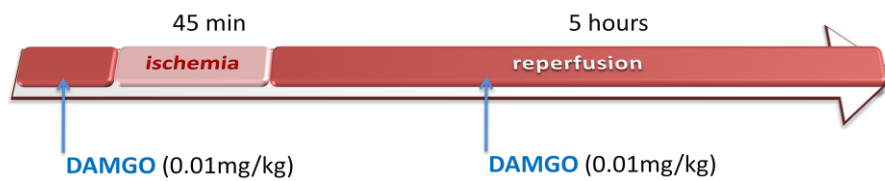
**Figure 13:** Doppler chart of small intestine bloodstream recorded continuously starting 10 min before the ischemia and ending after 10 min of reperfusion. Note the reduction of blood flow during ischemic period and the return of blood flow to normal values at the end of the reperfusion.

Three groups of 30-35 animals were investigated:

1. **I/R group:** mice underwent 45 minutes of intestinal ischemia followed by 5 hours of reperfusion;
2. **SO group:** mice underwent sham operation (same surgical procedure and intestinal manipulation of I/R group, without SMA occlusion);
3. **N group:** normal mice, which did not undergo any type of surgery.

Each group was randomly subdivided in four subgroups, each receiving one of the following subcutaneous treatments:

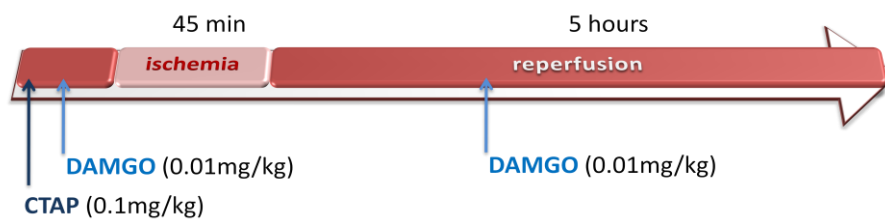
- I. Peripheral selective  $\mu$ OR agonist [D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin (DAMGO) injected subcutaneously at the dosage of 0.01 mg/kg, 20 min before the ischemic period and 2h after reperfusion starts.



- II. Peripheral selective  $\mu$ OR antagonist [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>] (CTAP) administered subcutaneously at the dosage of 0.1 mg/kg, 35 min before beginning of ischemia.



- III. Co-administration of DAMGO (0.01mg/kg s.c.) and CTAP (0.1 mg/kg s.c.) according with the previous scheduled time.



IV. Subcutaneously injection of the vehicle used for peptides administration: saline solution (NaCl 0.9%, 1ml/kg) 20 min before the beginning of ischemic period.



Dosages and administration schedules of  $\mu$ OR agonist and antagonist were chosen based both on scientific literature reported data<sup>95</sup> supported by a pilot study (DAMGO 0.01, 0.01 and 0.001 mg/kg; CTAP 0.1 and 0.01mg/kg), as well as considering elimination half-lives of the peptides (DAMGO:  $t_{1/2}$ =15 min<sup>97</sup>; CTAP:  $t_{1/2}$ >8 hours<sup>98</sup>).

At the end of the experiments mice were euthanized with isoflurane followed by thoracotomy. Specimens of the ileum were immediately collected and processed for histological and biochemical analysis.

## **Intestinal histology**

Specimens of the distal ileum were collected from the different groups of animals to determine the level of mucosal damage. Following overnight fixation in 10% formalin, samples of ileum were embedded in paraffin. Sections (4  $\mu\text{m}$  thickness) were stained with hematoxylin and eosin.

Microscopic histological damage score was evaluated by a person unaware of the treatments and was based on a semi-quantitative scoring system<sup>99</sup> in which the following features were graded:

- ✓ *epithelium damage*: 0 morphologically normal; 1 development of sub-epithelial space; 2 sub-epithelial space with moderate lifting of epithelial layer; 3 severe epithelial lifting.
- ✓ *inflammatory cells infiltration*: 0 absence of infiltrate or less than five cells; 1 mild infiltration to the lamina propria; 2 moderate infiltration to the muscularis mucosa and submucosa; 3 severe transmural infiltration involving the muscle layer.
- ✓ *extent of muscle thickening*: 0 normal; 1 moderate; 2 severe.
- ✓ *edema*: 0 no edema; 1 zone edema in lamina propria and submucosa; 2 diffuse edema in lamina propria and submucosa.
- ✓ *apoptotic cells in crypts*: 0 no or less than five apoptotic cells; 1 few apoptotic cells on some crypts; 2 diffuse apoptotic cells on crypts throughout the whole cross-section.

Data were expressed as sum of scores for each feature (total score).

## Gastrointestinal motility

Gastrointestinal (GI) transit was measured by evaluating the distribution of non-absorbable fluorescent 70,000MW dextran (150 $\mu$ l, 5mg/ml), administered intragastrically by gavages 90 minutes before the euthanasia of animals.



The entire GI tract, from stomach to distal colon, was excised and divided into 15 segments: stomach, small intestine (10 segments of equal length), cecum and colon (3 segments of equal length). The luminal content of each segment was collected, suspended in 1ml of distilled water, mixed vigorously and clarified by centrifugation (15min at 14,000rpm at 4°C). The supernatants of each sample were collected and the fluorescence signal was determined in triplicates by using a fluorescence plate reader (excitation wavelength 485nm and emission wavelength 528nm, FL<sub>X</sub>800 Microplate Fluorescence Reader, Bio-Tek instruments Inc., Winooski, VT, USA).

GI transit was calculated as the geometric centre (GC) of the distribution of labeled dextran along the entire gastrointestinal tract<sup>100</sup>.

$$GC = \sum(\% \text{ of fluorescence per segment}) \times (\text{number of segment})$$

GC value ranges from 1 (all the fluorescent dextran stays in the stomach, index of block in GI motility) to 15 (100% of the fluorescent signal measured in distal colon).



### **Myeloperoxidase activity**

Myeloperoxidase (MPO) activity, an index of tissue neutrophil accumulation, was measured according to Krawisz's modified method<sup>101</sup>.

Intestinal samples were homogenized (1:10, v/v) in a solution containing aprotinin 1µg/ml in 100mM potassium phosphate buffer (pH 7.4) and centrifuged for 25min at 10,000rpm at 4°C. Pellets were re-homogenized (1:5, v/v) in 50mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB) and aprotinin 1µg/ml. Samples were divided into two aliquots, subjected to three cycles of freezing (15min, -80°C) and thawing (20min, 37°C), and then centrifuged for 30min at 12,000rpm at 4°C. An aliquot of the supernatant (100µl) was allowed to react with a buffer solution of *o*-dianisidine (0.167mg/ml) and 0.0005% H<sub>2</sub>O<sub>2</sub>. The rate of change in absorbance was measured with a spectrophotometer at 470nm (Du® 530, Beckman Coulter, Fullerton, CA, USA).

One unit of MPO was defined as the quantity of enzyme degrading 1 mmol of peroxide per minute at 25°C. Data were expressed in mU/mg of wet tissue.

## **Cytokines and $\mu$ OR mRNA expression in the ileum**

### Total RNA extraction

Samples of ileum were homogenized with Power Gen 125 (Fischer scientific, Pittsburgh, PA, USA) and total RNA was extracted using the Absolutely RNA<sup>®</sup> RT-PCR Miniprep Kit (Stratagene, La Jolla, CA, USA). The quality of RNA was verified by electrophoresis on a 2% agarose gel and the amount of the purified RNA was determined by spectrophotometry (Nanodrop 1000, Thermo Scientific, Waltham, MA, USA). Spectrophotometric analysis of the samples showed absorption (OD) ratio  $OD_{260nm}/OD_{280nm} > 1.9$ , indicating excellent purity of the ribonucleic acids.

Total RNA (1  $\mu$ g) was reverse transcribed using SuperScript<sup>™</sup> III First-Strand Synthesis System for RT-PCR (Invitrogen Corp., Carlsbad, CA, USA).

### Real-time quantitative RT-PCR

The expression of  $\mu$ OR, pro-inflammatory cytokine TNF- $\alpha$  and interleukins 1 $\beta$  and 10 (IL-1 $\beta$  and IL-10) mRNA were assessed using quantitative real time reverse transcription-polymerase chain reaction (RT-PCR). Pre-designed mice TaqMan probes were used to quantify  $\mu$ OR, TNF- $\alpha$ , IL-1 $\beta$  and IL-10 gene expression (TaqMan<sup>®</sup> Gene Expression Assays, Applied Biosystem, Carlsbad, CA, USA). The PCR reaction mixture was incubated at 95°C for 10 min and then run for 50 cycles at 95°C for 15 sec and 60 °C for 1 min using Stratagene<sup>®</sup> Mx 3000p<sup>™</sup> machine. Expression of  $\beta$ -actin (an endogenous internal control gene) was measured in parallel for every sample and the data obtained for  $\mu$ OR, TNF- $\alpha$  and interleukins were normalized to those of the  $\beta$ -actin. Relative quantities (RQ) of mRNA were analyzed using the comparative threshold cycle ( $C_T$ ) method<sup>102, 103</sup>.

## **Drugs**

DAMGO ([D-Ala<sup>2</sup>, N-Me-Phe<sup>4</sup>, Gly<sup>5</sup>-ol]-Enkephalin), CTAP ([H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>]), *o*-dianisidine, HTAB, aprotinin were obtained from Sigma Aldrich Corporation (St. Louis, MO, USA). Myeloperoxidase was purchased from Calbiochem-EDM Chemicals, (La Jolla, CA, USA).

## **Statistic analysis**

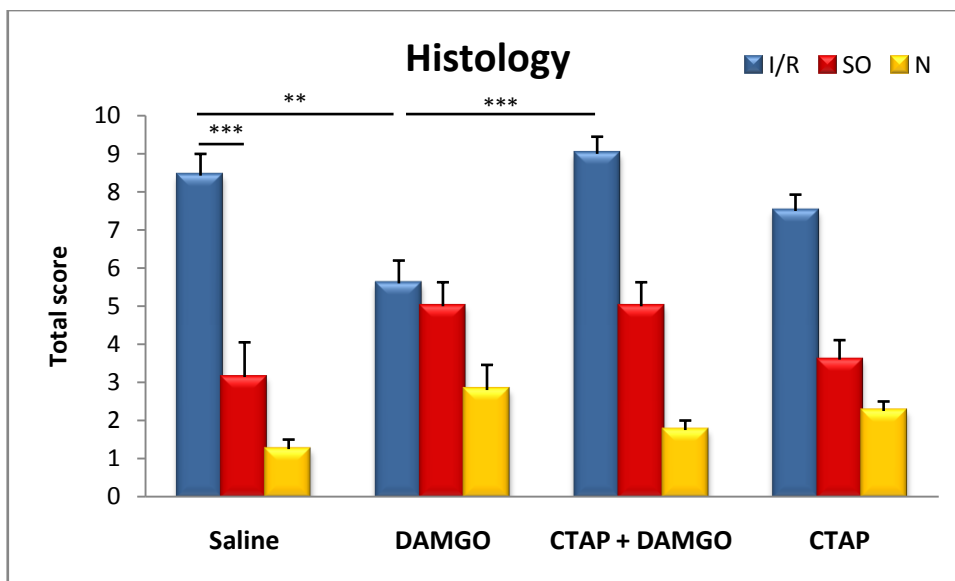
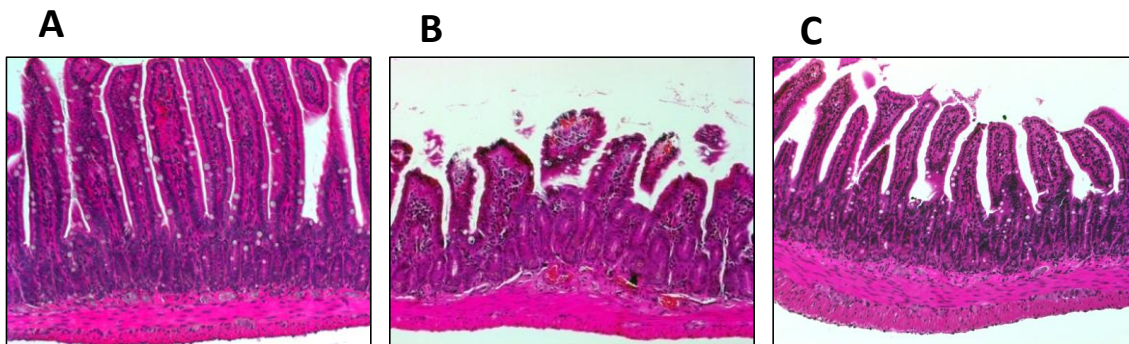
Data are expressed as means  $\pm$ SEM. Comparison among groups was made using analysis of variance (two-way ANOVA) followed by Bonferroni's post-test. Differences were considered significant when P value was below 0.05. Analysis was performed using Prism 4 program (GraphPad Software, San Diego, CA).

# RESULTS

## **Intestinal histology**

Sham operation induced edema and slight epithelial lifting compared to N mice. Among saline treated animals, an increase in the histological damage of I/R mice compared to SO and N was observed for each parameter considered and the total score was significantly increased (I/R =  $8.43 \pm 0.57$ ; SO =  $3.14 \pm 0.91$ ; N =  $1.25 \pm 0.25$ ;  $p < 0.001$ ). Intestinal I/R induced pronounced neutrophil infiltration within lamina propria and submucosa, edema, lifting of the epithelial layer from lamina propria, apoptosis in crypts cells and muscle thickening.

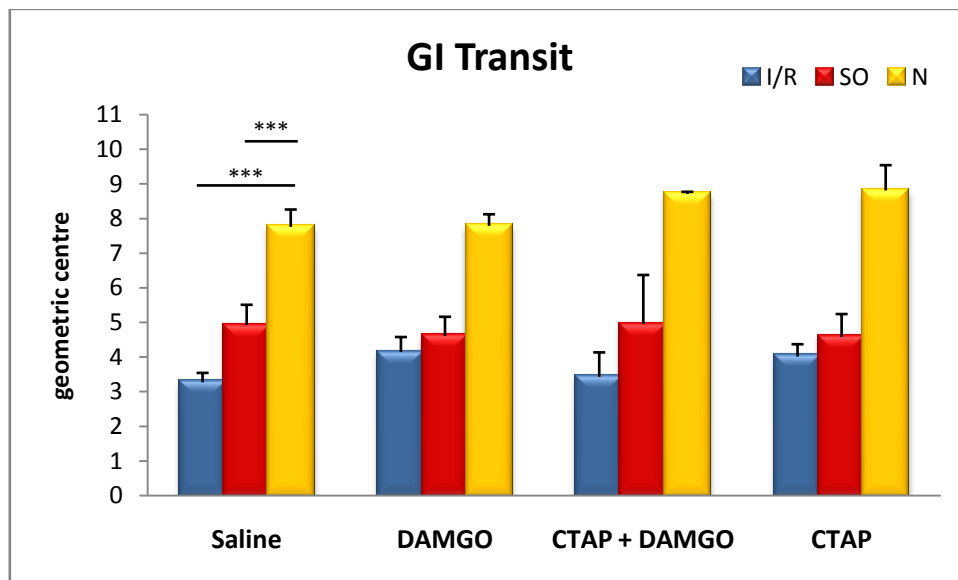
Treatment with the selective  $\mu$ OR agonist peptide DAMGO, significantly reduced histological ileal damage in I/R mice by lowering edema, epithelial lifting, number of apoptotic cells in crypts and infiltration of inflammatory cells in intestinal tissue (total score I/R treated with DAMGO =  $5.6 \pm 0.6$ ,  $p < 0.01$ ). This effect was completely reversed by the co-administration of the  $\mu$ OR antagonist peptide, CTAP (total score =  $9.0 \pm 0.45$ ,  $p < 0.001$ ). Treatment with  $\mu$ OR agonist or antagonist did not affect the histological appearance in the SO and N groups compared to vehicle treated animals.



**Graph 1:** Histological damage. Representative histological section of mice ileum from normal [A], and ischemic/reperfused [B] animals. I/R induced marked neutrophil infiltration, edema, epithelial lifting, apoptosis in cells in the crypts and muscle thickening. DAMGO significantly reduced histological damage in I/R mice [C] ( $p < 0.01$ ), effect that was reversed by co-administration of CTAP. Levels of tissue damage were evaluated using a semi-quantitative scoring system; final data are summarized in the histogram. All values are reported as mean  $\pm$  SE of 6-7 animals. \*\* $p < 0.01$ , \*\*\*  $p < 0.001$  (two-way ANOVA followed by Bonferroni's post-test).

## Gastrointestinal transit

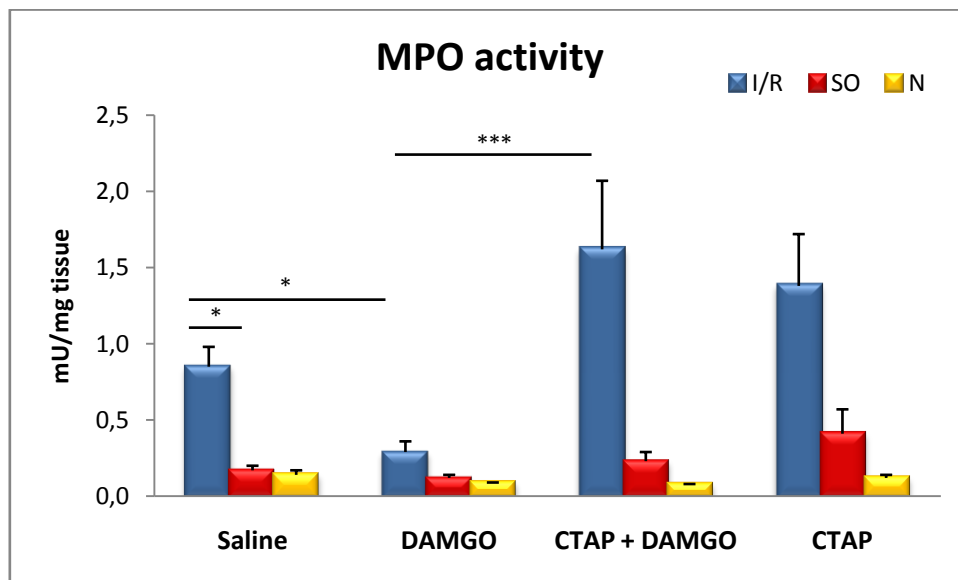
A significant delay in GI transit was observable both in I/R (geometric centre GC =  $3.27 \pm 0.27$ ) and in SO (GC =  $4.93 \pm 0.58$ ) groups compared to N (GC =  $7.76 \pm 0.50$ ) ( $p < 0.001$ ). There is slightly delay in GI transit between I/R and SO animals, however not statistically different. GI transit in I/R, SO or N was not affected by administration of DAMGO (GC =  $4.14 \pm 0.43$  in I/R group;  $4.61 \pm 0.55$  in SO group;  $7.79 \pm 0.33$  in N group) or CTAP (GC =  $4.01 \pm 0.36$  in I/R group;  $4.58 \pm 0.66$  in SO group;  $8.81 \pm 0.73$  in N group).



**Graph 2:** Effect on gastrointestinal motility in ischemic-reperfused, sham operated and normal mice with different treatments: saline solution (1ml/kg), DAMGO (0.01mg/kg), DAMGO+CTAP (0.1mg/kg) or CTAP alone. All values are reported as mean  $\pm$ SEM of 6-10 animals. \*\*\*  $p < 0.001$  (two-way ANOVA followed by Bonferroni's post-test).

## Myeloperoxidase activity

MPO activity in tissue homogenates was measured to assess the extent of neutrophils recruitment. I/R induced a 5-fold increase in intestinal MPO activity ( $p < 0.05$ ), compared to the SO and N animals (I/R =  $0.85 \pm 0.13$ ; SO =  $0.17 \pm 0.03$  and N =  $0.14 \pm 0.03$  mU/mg of wet tissue). DAMGO treatment resulted in a pronounced and significant reduction of MPO levels in I/R mice compared to saline (I/R =  $0.29 \pm 0.07$ ,  $p < 0.05$ ). DAMGO effect was completely reversed by simultaneous treatment with CTAP (I/R =  $1.62 \pm 0.45$ ,  $p < 0.001$ ), confirming that it was a receptor-mediated effect. CTAP alone induced a slight, but not significant, increase of MPO activity in I/R compared to I/R group treated with saline (I/R =  $1.38 \pm 0.34$ ) and a weak increase in SO mice compared to SO treated with saline (SO =  $0.41 \pm 0.16$ ). DAMGO or CTAP administration did not affect MPO activity in N mice.



**Graph 3:** MPO activity, index of neutrophil accumulation in intestinal tissue, in ischemic-reperfused, sham operated and normal mice treated with: saline solution (1ml/kg), DAMGO (0.01mg/kg), DAMGO+CTAP (0.1mg/kg) or CTAP alone. All values are reported as mean  $\pm$ SE of 6-10 animals. \*  $p < 0.05$ , \*\*\*  $p < 0.001$  (two-way ANOVA followed by Bonferroni's post-test).



## **Cytokines mRNA expression**

### Interleukin-1 $\beta$ (IL-1 $\beta$ )

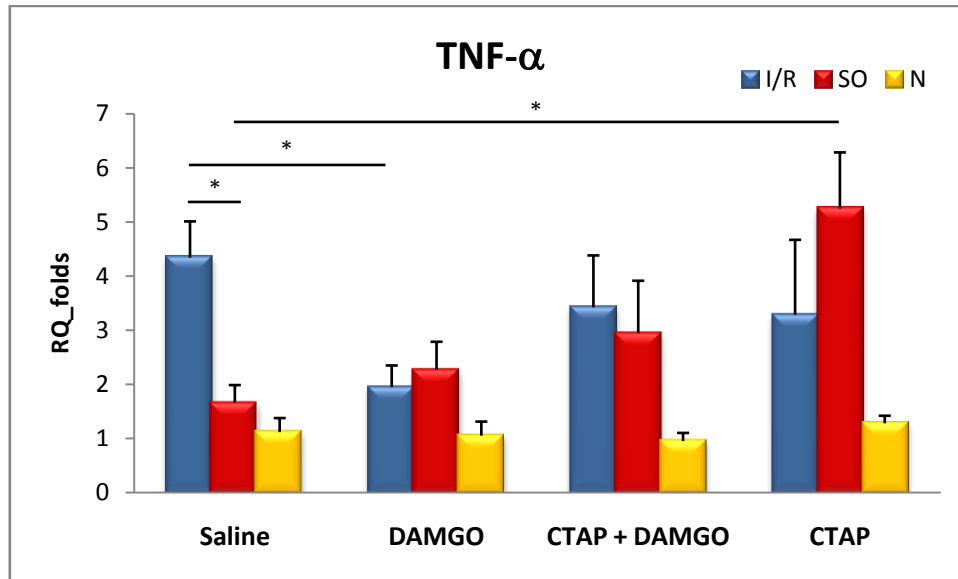
IL-1 $\beta$  is a cytokine released from macrophages able to promote the inflammatory response, increasing endothelial leukocyte adhesion and phagocyte activation<sup>104</sup>.

The mRNA levels of IL-1 $\beta$  quantified in ileum tissue of I/R (RQ\_folds = 2.36 $\pm$ 0.44) and SO (2.47 $\pm$ 0.46) mice were little higher compared to N (1.10 $\pm$ 0.17) animals, however there were no significant differences in tissue expression of this cytokine.

### Tumor Necrosis Factor- $\alpha$ (TNF- $\alpha$ )

TNF- $\alpha$  is a pro-inflammatory cytokine, released from macrophages, monocytes, lymphocytes and other cells, capable of increasing other cytokines release in the inflammatory cascade as well as eliciting leukocyte migration<sup>104</sup>.

The levels of TNF- $\alpha$  mRNA expression quantified by real time PCR were significantly higher in I/R animals compared to SO and N mice (RQ\_folds I/R = 4.34 $\pm$ 0.67, SO = 1.66 $\pm$ 0.33 and N = 1.12 $\pm$ 0.26; p<0.05). Treatment with DAMGO inhibited the production of TNF- $\alpha$  mRNA in I/R mice (I/R = 1.95 $\pm$ 0.40; p<0.05) and this effect was abolished by the co-administration of CTAP (I/R = 3.42 $\pm$ 0.96). CTAP alone did not modify the expression of TNF- $\alpha$  mRNA in I/R mice but significantly increased it in SO (SO = 5.25 $\pm$ 1.04; p<0.05), suggesting the occurrence of endogenous stimulation of  $\mu$ ORs in the modulation of this cytokine pathway during surgery manipulation. TNF- $\alpha$  mRNA in N mice was not altered by DAMGO or CTAP administration.

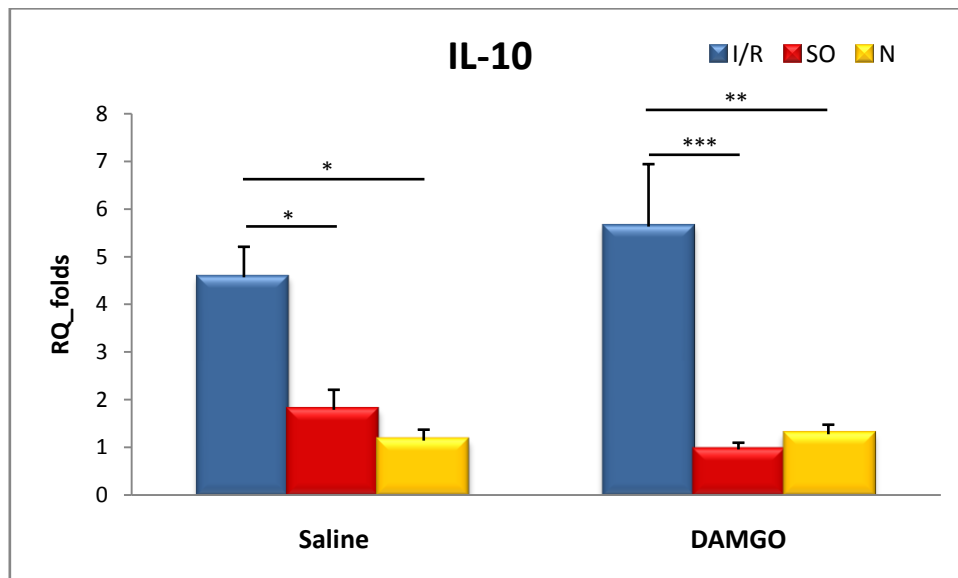


**Graph 4:** Quantification of TNF- $\alpha$  mRNA concentrations in intestinal tissue of ischemic-reperfused, sham operated and normal mice treated with: vehicle (saline solution, 1ml/kg), DAMGO (0.01mg/kg), DAMGO+CTAP (0.1mg/kg) or CTAP alone. All values are reported as mean  $\pm$ SE of 8-10 animals. \*  $p < 0.05$  (two-way ANOVA followed by Bonferroni's post-test).

## Interleukine-10 (IL-10)

IL-10 is a counter regulatory cytokine produced by T-helper2 cells, implicated as an inhibitor of pro-inflammatory cytokine production and of several accessory cell functions of the macrophage, T-cells and NK cell lines<sup>104</sup>.

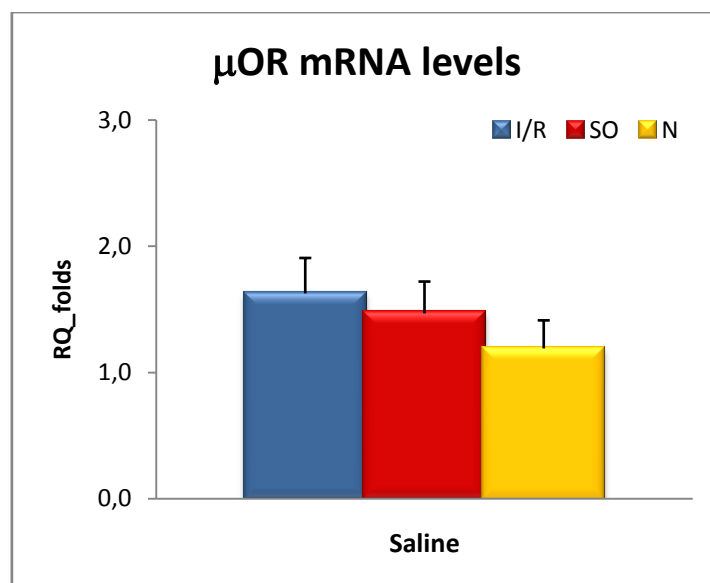
IL-10 mRNA expression was significantly higher in I/R mice (RQ\_folds =  $4.57 \pm 0.64$ ) compared to SO ( $1.79 \pm 0.42$ ;  $p < 0.05$ ) and N animals ( $1.14 \pm 0.23$ ;  $p < 0.05$ ). However, the treatment with DAMGO did not alter the expression of this cytokine (I/R =  $5.63 \pm 1.31$ , SO =  $0.96 \pm 0.14$ , N =  $1.27 \pm 0.2$ ).



**Graph 5:** Quantification of IL-10 mRNA concentrations in intestinal tissue of ischemic-reperfused, sham operated and normal mice treated with: vehicle (saline solution, 1ml/kg) and DAMGO (0.01mg/kg). All values are reported as mean  $\pm$ SE of 8-10 animals. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  (two-way ANOVA followed by Bonferroni's post-test).

## $\mu$ OR mRNA levels

Recent evidence showed that intestinal inflammation induces an increment in  $\mu$ OR expression. In our experimental model and conditions, we did not observe significant differences in  $\mu$ OR mRNA levels in the ileum samples of I/R, SO and N mice. (RQ\_fold  $1.63 \pm 0.28$  in I/R group;  $1.47 \pm 0.25$  in SO group;  $1.19 \pm 0.22$  in N group).



**Graph 6:** Quantification of  $\mu$ OR mRNA expression in intestinal tissue of ischemic-reperused, sham operated and normal mice treated with vehicle (saline solution, 1ml/kg). All values are reported as mean  $\pm$ SE of 8-10 animals. There are no significant differences between the

# **DISCUSSION**

An experimental model of mesenteric ischemia and reperfusion in rats and mice was previously set up by our group<sup>28, 44, 96</sup>. In this model, occlusion of the superior mesenteric artery (SMA), through microvascular clips or nylon threads, was performed for 45 minutes and followed by 24 hours of reperfusion. These experimental conditions were associated to a mild intestinal inflammation and reversible changes in enteric motility<sup>96</sup>. During reperfusion, PMNs are activated and start to infiltrate into the bowel wall, contributing to the tissue injury typical of acute inflammatory response<sup>105</sup>. In this new investigation we choose a reperfusion time of 5 hours based on previous studies showing that the maximal systemic activation of neutrophils occurs within 4-5 hours of reperfusion<sup>106</sup>. In this experimental conditions (I45'/R5h) animals underwent ischemia and reperfusion showed increased mucosal damage characterized by pronounced neutrophil infiltration within lamina propria and submucosa, edema, lifting of the epithelial layer from lamina propria, apoptosis in crypts cells and muscle thickening. Neutrophils infiltration in intestinal tissue was supported also by the increase of MPO activity measured in ileal homogenates. Moreover, the presence of inflammation was confirmed by the augmented expression of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-10) mRNA in I/R mice; while a delay in the gastrointestinal transit was detected as index of impaired gastrointestinal motility.

Derangement of functional communication between enteric nerves with hormones and neuropeptides, like opioid peptides, has been shown to play a major role in the pathophysiology of Inflammatory Bowel Diseases (IBD)<sup>107</sup>; and it is demonstrated that exogenous specific activation of  $\mu$ OR exerts a protective effect controlling inflammation induced by experimental colitis in rodents, even if the exact mechanism underlying is not completely clarified<sup>94, 95</sup>. This study displays that the exogenous

activation of  $\mu$ ORs, through the selective agonist DAMGO, prevents intestinal injuries associated with mesenteric I/R in mice. Indeed, DAMGO treatment ameliorates histological damage of the mucosa by reducing inflammatory cells infiltration within lamina propria and submucosa, edema, epithelial lifting, presence of apoptotic cells in crypts and muscle thickening in I/R mice. It is demonstrated that intestinal I/R is associated with the early recruitment of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells and T-cells seem to modulate the infiltration of neutrophils that occurs hours after the inception of reperfusion<sup>8, 23</sup>. This study shows that DAMGO administration was able to reduce the inflammatory response caused by mesenteric I/R and that  $\mu$ ORs activation strongly decreases neutrophil accumulation in intestinal tissue, as attested from the drop in MPO activity compared to untreated I/R mice.

In order to understand the underneath mechanism of  $\mu$ OR protective effect we analyzed the intestinal expression of different cytokines. TNF- $\alpha$  is a pro-inflammatory cytokine, released from macrophages, monocytes, lymphocytes and other cells, capable of increasing other cytokines release in the inflammatory cascade as well as eliciting leukocyte migration<sup>104</sup>. An increase of the pro-inflammatory cytokine TNF- $\alpha$  levels has been reported during the ischemic period and the early reperfusion in mice<sup>108</sup> and our data, in accordance, demonstrate that after 5 hours of reperfusion, TNF- $\alpha$  levels are significantly augmented in post ischemic intestinal tissues and give evidence that mRNA expression of TNF- $\alpha$  is reduced after  $\mu$ ORs activation with DAMGO. Interleukin 1 $\beta$  (IL-1 $\beta$ ) is a cytokine released from macrophages that promotes the inflammatory response, increases endothelial leukocyte adhesion and phagocyte activation<sup>104</sup>. The levels of IL-1 $\beta$  expression in the ileum of I/R and SO mice were lightly increased compared to N animals, however there was no significant difference

between ischemic and sham operated animals. On the other hand, we analyzed the expression of interleukin 10 (IL-10), a counter regulatory cytokine produced by T-helper2 cells, implicated as an inhibitor of pro-inflammatory cytokines production and of several accessory cell functions of the macrophage, T-cells and NK cell lines<sup>104</sup>. Our data showed that IL-10 mRNA expression was significantly higher in I/R mice compared to SO and N; however, treatment with DAMGO did not modify this expression pattern. Based on these findings we suggest that the anti-inflammatory role of  $\mu$ OR is likely correlated with regulation of cytokines expression, such as TNF- $\alpha$ , and the consequent modulation of inflammatory cells recruitment.

In order to assess if DAMGO protective effects on histological damage score, MPO activity and TNF- $\alpha$  mRNA expression were  $\mu$ ORs mediated, we repeated the experiment treating the animals with DAMGO in presence of the selective  $\mu$ OR antagonist CTAP. Data obtained showed that beneficial effects of DAMGO, on each inflammatory index checked, were abolished by simultaneous administration of the selective  $\mu$ OR antagonist CTAP; confirming that they were  $\mu$ ORs mediated.

Previous study showed that  $\mu$ OR mRNA expression is increased in inflammatory bowel disease in humans and in experimental TNBS-induced colitis in mice<sup>95</sup> and in a model of croton-oil induced intestinal inflammation<sup>93</sup>. Our results did not display any increase in the mRNA receptor expression. A possible explanation for these data could be associated with the relatively short time (5 hours) that occurs between the I/R injury and the euthanasia of the mice. It is likely that a chronic inflammation or a more severe and persistent mucosal damage are necessary to activate intracellular pathways able to increase  $\mu$ OR gene transcription.



It is well known that opioid peptides have a physiological role in the control of gut motility and that opiate  $\mu$ ORs stimulation induces a delay in gastrointestinal transit. In this study administration of DAMGO did not modify the GI transit that appeared reduced in I/R and SO compared to N mice. The inability to reveal DAMGO effect on GI transit, even in N animals, is probably due to the length of the interval of time that elapses between DAMGO administration and GI transit measurement performed in this study (about 3 hours). The short half life of DAMGO ( $t_{1/2} = 15$  min) accounts for the repeated DAMGO administration we adopted in this study and leads us to interpret DAMGO effects, when present, as indirect rather than direct responses. Reasonably DAMGO effects are consequence of interference with the processes operating during ischemia and reperfusion rather than the direct presence of the agonist at the moment of parameter measurement. In fact, previous studies on rats showed that the inhibition of GI transit can be appreciated only within 25 minutes from DAMGO systemic administration<sup>109</sup>. On the other hand, the absence of any effect of the  $\mu$ OR antagonist CTAP treatment on motor activity in each experimental group suggests a negligible involvement of endogenous opioid system in the GI transit delay observed in SO and I/R mice.

Opiate-mediated immunomodulatory effects have been postulated to result from direct activation of opioid receptors on cells of the immune system or indirectly, through the activation of opioid receptors within the central nervous system and the resulting modulation of hypothalamic pituitary adrenal axis and the sympathetic nervous system<sup>67</sup>. Both DAMGO and CTAP are not able to cross the blood-brain barrier suggesting that anti-inflammatory properties shown in this experimental model are correlated to the activation of peripheral  $\mu$ OR and are not related to stimulation of

central receptors. These findings support the hypothesis that peripheral  $\mu$ OR agonists could have therapeutic anti-inflammatory effects in intestinal ischemia, without appearance of unwanted central adverse effects (e.g. respiratory depression, euphoria, somnolence, sedation and addiction)<sup>110</sup>. Furthermore the participation of the endogenous opioid system and its relevance in the modulation of the immuno-response activated by intestine manipulation is supported by the significant increase of TNF- $\alpha$  mRNA levels observed after CTAP treatment in SO mice. The absence of such effect in I/R mice could simply derive from the simultaneous activation of other additional biochemical pathways, that cooperate along the opioid system, in the physiological counter-regulation of the more sustained acute inflammatory response elicited by ischemia and reperfusion. In this context opioid system can be seen as an early endogenous regulator involved in the pro-resolving mechanism aimed at blunting the TNF- $\alpha$  production, whose increase is the initial sub-clinical response to manipulation of the intestine.

In summary, this investigation shows that exogenous activation of  $\mu$ ORs plays a protective role in our experimental model of mesenteric ischemia-reperfusion in mice. Indeed,  $\mu$ OR activation is able to reduce the intestinal damage induced by ischemia and reperfusion, through a mechanism that appears to be in part mediated by TNF- $\alpha$ . This suggests that peripheral  $\mu$ OR agonists might be potential therapeutic approaches for I/R-induced inflammatory injury.

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