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EXPERIMENTAL STUDY

The protective role of anandamide in mesenteric ischemia reperfusion injury in guinea pig

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Abstract: *Background:* Acute mesenteric ischemia is an entity characterized by rapid developing of circulatory failure. Reperfusion following ischemia causes further mucosal injury.
Methods: In our study, an experimental model of 15 minutes of reperfusion following 45 minutes of superior mesenteric artery occlusion was established. The segments which underwent I/R injury were histopathologically examined, and blood samples obtained from the heart were analyzed for alkaline phosphatase and creatine kinase levels.
Results: The results of the study demonstrated that mucosal injury in anandamide injected group was less expressed than in other groups suggesting that anandamide might have a protective effect on the mucosa. After L-NAME and indomethacin injection, the protective effect of anandamide seems to disappear due to inhibition of NO and prostaglandins. The results of histopathological examination of specimens from CB1 receptor and anandamide injected group indicate that I/R injury has regressed.
Conclusion: The protective effect of endogenous anandamide on I/R injury may take place through CB2 receptors in the small intestine; NO and prostaglandin, which are activated through the stimulation of CB2 receptors may be responsible for this protective effect *(Fig. 8, Ref. 29)*. Full Text (Free, PDF) *www.bmj.sk.*
Key words: anandamide, ischemia – reperfusion, AM251, L-NAME, rat.

Acute mesenteric ischemia arises as a rapid onset of circulatory failure, and inadequate perfusion of abdominal organs leads to various metabolic changes. The mortality rate in patients with acute mesenteric ischemia varies between 60 to 100 % (1).

Acute ischemia of mesenteric organs may result from systemic diseases and/or local factors as well as low flow. Neutrophil infiltration towards the tissue during reperfusion phase, accumulating oxygen radicals, lipid peroxidation and degradation due to several enzymes cause breakdown of cellular functions and cell necrosis (1, 2).

The debate on the pathophysiological basis of mesenteric ischemia reperfusion (I/R) injury still goes on. In this study, the effect of anandamide (arachidonoylethanolamide) – an arachidonic acid metabolite – on I/R injury was investigated as well as the roles of CB1 and CB2 receptors.

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Materials and method

The study was carried out at Gazi University Experimental Research Laboratory. All permissions were obtained from Gazi University Ethical Committee. Thirty-six guinea pigs with mean weight of 450 g (350–550 g) were used. There were six groups with six subjects in each. They were restricted for food 12 hours before the experiment with only free access to water. Intramuscular ketamine hydrochloride (Ketalar, Parke Davis, Istanbul, Turkey 50 mg/mL) was used for anesthesia in a dose of 60 mg/kg. In I/R groups, a 15-minute reperfusion followed a 45-minute period of ischemia (3).

Groups

I: Sham group. Only laparotomy was performed and the superior mesenteric artery (SMA) was found.

II: I/R group. After laparotomy, ischemia and reperfusion were performed taking 45 and 15 minutes, respectively.

III: Anandamide group. Laparotomy was performed. After the injection of 0.1 ml of 10^{-6} molar Anandamide (Tocris, Northpoint, Fourth Way, Avonmouth, Bristol, UK) via the SMA, I/R was performed.

IV: Anandamide + L-NAME group. 10^{-4} molar L-NAME (NG-Nitro-L-Arginine Methyl Ester) (Sigma Chemical Co, St. Louis,

USA) was administered 10 min before laparotomy. After laparotomy, 10^{-6} molar Anandamide was administered 0.1 ml via the SMA followed by I/R.

V: Anandamide + Indomethacine group. 10^{-6} molar Indomethacine (Sigma Chemical Co, St. Louis, USA) was administered 10 min before laparotomy. After laparotomy, 10^{-6} molar Anandamide was administered 0.1 ml via the SMA followed by I/R.

VI: Anandamide + AM251 (CB1 receptor antagonist) group. After laparotomy, 0.1 ml 10^{-6} molar AM251 – CB1 receptor antagonist (N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-5-(4-chlorophenyl)-4-m ethyl-1H-pyrazole-3-carboxamide) (Tocris, Northpoint, Fourth Way, Avonmouth, Bristol, UK) was administered. After that, 0.1 ml 10^{-6} molar Anandamide was administered via the SMA followed by I/R.

Surgical technique

After local cleansing, a midline laparotomy was performed. Superior mesenteric artery was occluded by microclamps as well as the terminal branches to the proximal jejunum. The pharmacological agents were administered using insulin injectors. After reperfusion, ischemic intestinal segments were resected for pathologic examination. At the end of the experiment, blood samples were taken from the heart transdiaphragmatically for alkaline phosphatase (ALP) and creatine kinase (CK) levels. The tested subjects were sacrificed by creating hypovolemia.

Pathological examination

The resected jejunal segments were opened along the antimesenteric side and fixed for 12 hours in 10% formalin solution. After deparafinization, they were stained using hematoxilene-eosine. Ischemic injury was scaled according to the classification by Park and Chiu (4).

Biochemical evaluation

Blood samples were examined in terms of ALP and CK levels with a Roche kit (Roche ALP Kit, GmbH D-68928 Mannheim, Germany) in a Roche apparatus (Roche Hitachi 912, Germany) calorimetrically. Results were given as U/L.

Statistical analysis

A commercially available program, SPSSr 11.5 (Standard Package of Statistical Sciences) was used. One-way Variance Analysis, Kruskal-Wallis and Mann-Whitney U tests were used for pathological injury assessment while Kruskal-Wallis and Chi-Square tests were used for blood sample analysis. Values less than 0.05 were accepted to be significant.

Results

A statistically significant difference was observed between groups in terms of mucosal damage due to ischemia and reperfusion. Mean pathological grades of the groups were 0, 2.50, 2.17, 2.50, 2.83 and 0.33, respectively. These values are shown in Figure 1. CB1 receptor antagonist group (Group VI) had the least pathological mucosal damage. There was a statistically significant difference between the control groups and the other test groups ($p<0.005$).

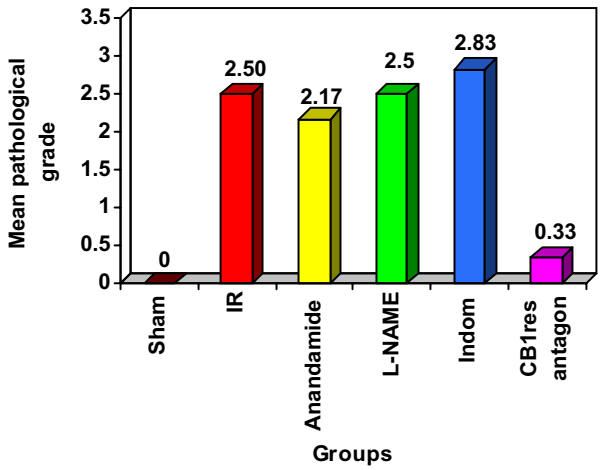

Fig. 1. Mean pathological damage grades of groups.

There was no ischemic damage in the sham group (Group I). Mucosa and villi structures were normal (Grade 0) (Fig. 2). In I/R group (Group II), three guinea pigs had Grade 2 damage while the other three had Grade 3 (Fig. 3). In the I/R+Anandamide group (Group III), Grade 3 damage in one guinea pig and Grade 2 in the remaining five was seen (Fig. 4).

The highest grades for ischemic damage (Grade 4) were observed in Anandamide+L-NAME (Group IV) and Anandamide+Indomethacine group (Group V) (Fig. 5). In Anandamide+CB1 receptor antagonist group (Group VI), the highest ischemia grade was Grade 1 and this was observed in two guinea pigs only. No ischemia was observed in the remaining four guinea pigs of this group (Fig. 6).

The ALP and CK levels of the groups are shown in Figures 7 and 8. There was a significant difference between the CK values of the groups I and II, III, IV, and V ($p=0.004$). No difference was found between group I and the group VI. A significant difference was found between the ALP levels of group I with those of groups II and III ($p=0.016$). However, no difference was found between group I and groups IV, V and VI.

No statistically significant difference was found between group II and groups III, IV and V in terms of ALP and CK values. However, there was a significant difference in these variables between groups II and VI ($p=0.004$ and $p=0.006$, respectively).

There was no significant difference between group III and group IV in terms of ALP value. On the contrary, there was a significant difference between group III and groups V and VI in terms of ALP value ($p=0.037$ and $p=0.006$, respectively). There was no statistically significant difference between CK values of group III and groups IV, V. However, there was a significant difference between groups III and VI, with regard to CK value ($p=0.025$).

There was no statistically significant difference between ALP and CK enzyme values of group IV and the ALP and CK enzyme values of group V. However, there was a significant differ-

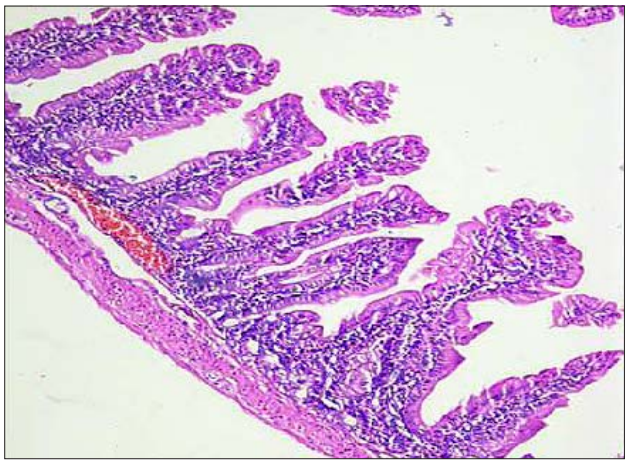

Fig. 2. Small intestinal mucosa showing non-ischemic (Grade 0), normal villi, Sham group (H&E x100).

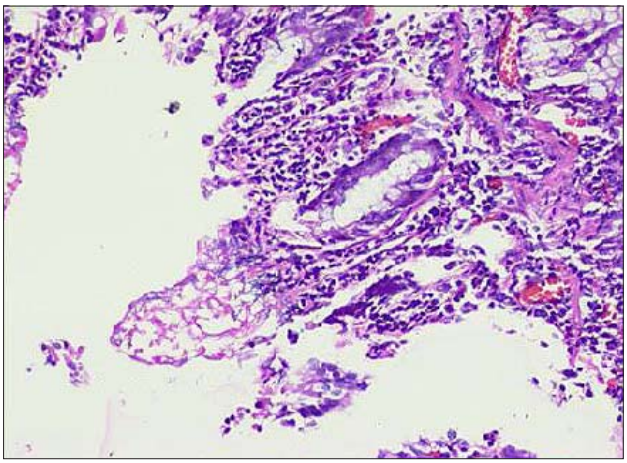

Fig. 5. Grade 4 ischemia characterized by denuded villi, disintegration of lamina propria and congestion, anandamid + L-NAME group and anandamid + indomethacine group (H&E x 200).

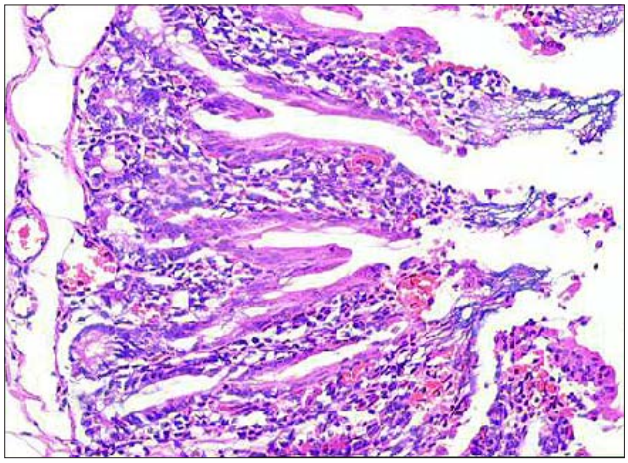

Fig. 3. Grade 3 ischemia chacterized by some denuded villi tips and exudation, ischemia-reperfusion group (H&E x 200).

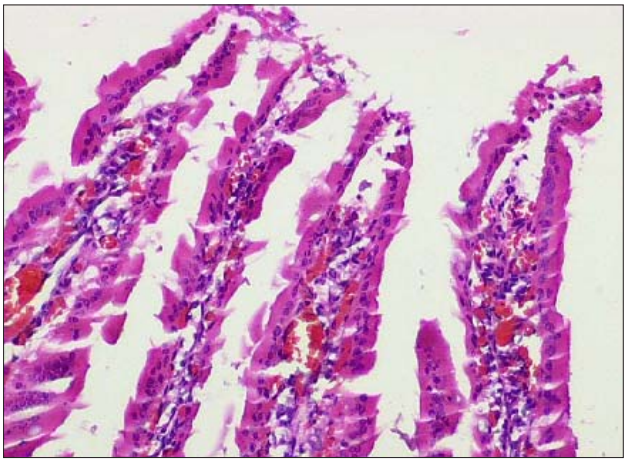

Fig. 6. Subepithelial space seen only at villus tips characterizing grade 1 ischemia; anandamide+CB1 receptor antagonist group (H&E x 400).

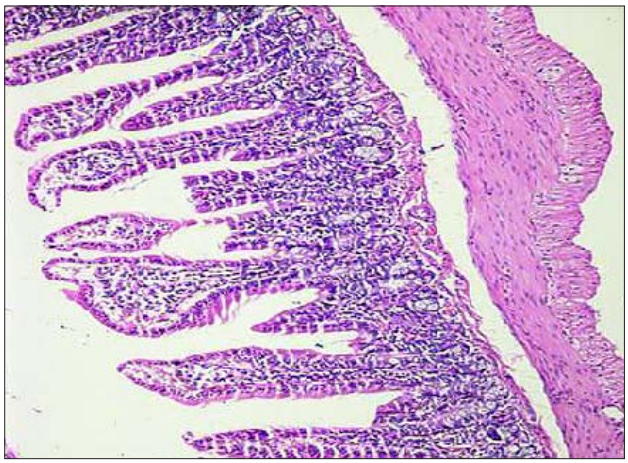

Fig. 4. Extension of subepithelial space, resulting from detachment of lamina propria from epithelium, from villus tips to down sides of villi, grade 2 ischemia, ischemia-reperfusion + anandamid group (H&E x 100).

ence between group IV and group VI, with regard to ALP and CK enzyme values (p=0.008, and p=0.016, respectively).

No statistically significant difference was found between groups V and VI with regard to ALP values. However, there was a significant difference between their CK values (p=0.01).

Discussion

Acute mesenteric ischemia is a clinical entity that occurs as a result of a sudden or rapidly developing circulatory failure. The degree of mucosal injury increases during reperfusion period following intestinal ischemia (5). The mesenteric collateral circulation in animals is very similar to that in humans. Recent studies have demonstrated that superior mesenteric artery (SMA) occlusion alone reduces the mesenteric flow by an average of 83 %. Intestinal ischemia models performed on rats by Megison et al. have demonstrated that, in order to form a continuous deep ischemia model, it is also necessary to stop the collateral flow, in

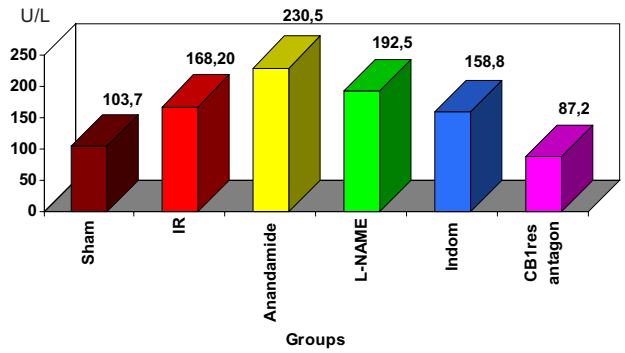

Fig. 7. ALP levels of the groups.

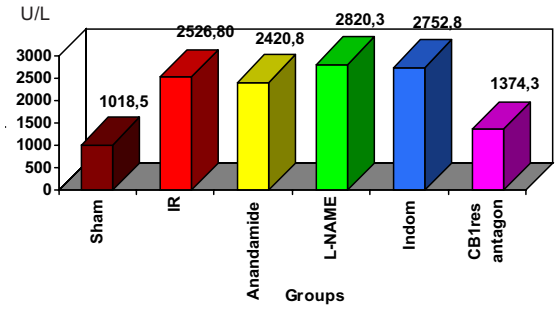

Fig. 8. CK levels of the groups.

addition to SMA occlusion (6). In this study, ischemia was produced by stopping mesenteric collateral flow, in addition to SMA occlusion, identically to the ischemia models described in literature.

Reconstruction of blood flow following ischemic events results in two important and positive effects on ischemic tissue; re-establishment of energy supply, and clearance of toxic metabolites. Reperfusion is a required event to repair the ischemic injury. In addition, inclusion of toxic substances into the systemic circulation may lead to severe metabolic consequences as well as to future increase in severity of local tissue damage caused by reperfusion. The formation of reactive oxygen species causes reperfusion (reoxygenation) injury in ischemic tissues (7, 8).

Many arachidonic acid metabolites cause splanchnic vasodilatation. Contrarily, PGF2α, PGB2, PGD2 prostaglandins, leukotriene C4 and D4 and some tromboxane analogs cause splanchnic vasoconstriction. Prostaglandins synthesis inhibitors such as aspirin, indomethacin and meclofenamate regulate the vasodilatation effect of one or more arachidonic acid metabolites in the splanchnic region (5).

ALP is a zinc-containing metalloglycoprotein type of dimeric enzyme of type-1 phosphomonoesterases that hydrolyzes various phosphate esters. In addition to being found in many cells, these enzymes are abundant in bone osteoblasts, hepatobiliary tract cells, intestinal mucosa, renal tubules, brain and placenta, being excreted through bile. CK is an enzyme originating in heart, skeletal muscle and brain tissue. It catalyzes the transfer of phosphate to creatine from adenosine triphosphate (ATP) (9, 10).

Nitric oxide (NO) antagonists have been used to investigate NO deficiency under oxidative stress conditions. The most commonly used substance in this category is L-NAME (11, 12).

Indomethacin is an agent with analgesic, antipyretic and anti-inflammatory effects. In experimental investigations, it has been demonstrated to prevent increased capillary permeability caused by various endogenous substances. In addition, they inactivate cytotoxic-active oxygen radicals through binding. It strongly inhibits phosphodiesterase in-vitro and increases the concentration of intracellular cyclic adenosine mono phosphate (cAMP). As a result, cAMP increased in cells with indomethacin may contribute to the anti-inflammatory effect (13, 14).

Marijuana has been used for centuries as both, an amusement substance that increases psychoactivity in human population as well as a medication. The therapeutic application of marijuana has been recorded as early as in the 4th century BC. By the mid 20th century, synhexyl (parahexyl), a substance that is similar to the active component of marijuana, was discovered (15, 16). In 1960, the synthetic active substance of 9-tetrahydrocannabinol (THC) was isolated by Israeli pharmacochemists, Y. Gaoni and R. Mechoulam (17). THC demonstrates the analgesic, antiemetic, anti-inflammatory, bronchodilative and antiepileptic effects of marijuana (15, 18). Since then, more than 300 pharmacologic agents, coined as cannabinoids, have been identified as a combination of THC and various similar unsaturated fatty acids as well as arachidonic acid with ethanolamine. Some endogenous binding agents, which respond to cannabinoid receptors, such as anandamide, 2-arachidonyl glycerol (2-AG) and 2-arachidonyl-glyceryl ether (2-AGE, noladin ether) have been identified (15, 18, 19). Cannabinoid receptors have been designated as CB and arranged in order of discovery. Recently, two cannabinoid receptors, CB1 and CB2, have been detected. They are differentiated according to their amino acid composition, signaling mechanism, and distribution in tissues (19, 20).

The degree of intestinal ischemia-reperfusion injury is routinely confirmed by histological analysis (4). In this study, the degree of injury was graded in the ischemia-reperfusion group as 2.50+0.55, according to the Park/Chiu system. As previously mentioned, the ischemic period lasted 45 minutes whereas the reperfusion period lasted 15 minutes in our ischemia-reperfusion model through occlusion of SMA supplemented by collateral flow prevention. The scores obtained in this study are higher than ischemia-reperfusion scores found in literature. In the study conducted by Jacob et al., the ischemia-reperfusion duration was found to be longer, whereas the score was found to be lower (21). On the other hand, Nilson et al. reported ischemia-reperfusion durations and histopathological results that were similar to those of our study (22).

Previous investigations have disclosed the role of endogenous opioid peptides in the prevention of intestinal ischemic injury (23). The effects of anandamide – an endocannabinoid synthesized from arachidonic acid – on the gastrointestinal tract have

been assessed in several studies and there are reports that they may be mediated through CB1 and CB2 receptors (16, 18, 24, 25). The effect of anandamide and analogous agents on tissue I/R injury has also been demonstrated in isolated cardiac models (26).

In this study, a histopathologic score of 2.17±0.41 was determined in one group before ischemia despite intra-arterial anandamide administration. In other words, a possible protective effect should be considered due to the smaller decrease in I/R score (2.50±0.55) compared to I/R group.

The NO synthase (NOS) inhibitor, L-NAME, inhibits both Ca^{2+}-dependent neuronal and endothelial NOS, and also non-Ca^{2+}--dependent NOS. Luo et al demonstrated that L-NAME decreases I/R injury by inhibiting endogenous NO (12).

In this study, the degree of I/R injury was found to be high (2.50+0.84) in the group that received L-NAME prior to anandamide. Administration of this inhibitor to animals will stop or reduce NO synthesis and reverse all functions where NO is required. It is able to increase the severity of intestinal injury and disables the protective effect of anandamide when it reaches the irreversible state. Takahashi et al reported that NO plays a role in motility regulation, blood flow, and oxygen consumption, as well as that L-NAME increases the injury degree under hypoxic conditions caused by ischemia (27).

The protection from ischemic damage during ischemia and subsequent reperfusion is due to the release of free oxygen radicals, thromboxane A2 and leukotrienes from tissues by prostaglandins and prostaglandin-like substances, instead of a direct cytoprotective effect. Cyclooxygenase inhibitors, such as indomethacin, inhibit the release and metabolism of these compounds (5, 13, 14). In the group that received indomethacin prior to anandamide, the pathologic degree of I/R injury was the highest. It was observed that the injury degree resulted from intestinal ischemia, and that the subsequent reperfusion increased after indomethacin administration. Sare et al. demonstrated that administration of indomethacin before laparotomy in an ischemia-reperfusion model inhibits prostaglandin E2 activity and therefore does not prevent intestinal vascular permeability (3).

In tissue, anandamide has a higher affinity for CB1 receptors (19, 28). In this study, the synthetic CB1 receptor antagonist, AM251, was administered prior to ischemia, whereas anandamide was later injected into the mesenteric circulation. Ischemia developed in all animals after drugs administration. The ischemic symptoms included pale intestinal wall and color darkening. Light-brown coloration of the macroscopic ischemic segment was observed while waiting for peristalsis to disappear, and wall edema was observed as peristalsis was in motion. In pathologic analysis, there was a first-degree injury in the ischemic intestinal segment of only two subjects, whereas no injury was found in the others. We believe that the protective effect of anandamide can be provided through CB2 receptors due to blockage of CB1 receptors.

In the study conducted by Fujino et al, CK values were markedly high, indicating intestinal injury (29). Blood enzyme measurements performed in our study was found to be significantly high in all groups, except for group I and group VI. Nevertheless, no statistically significant difference was detected amongst groups II, III, IV and group V. There is an increase in tissue enzymatic activity during ischemia and reperfusion following the administration of anandamide, L-NAME and indomethacin. However, our results suggest that this enzymatic increase does not reflect the injury degree or medication effect, and does not have any diagnostic value. Sare et al. also obtained similar results in their I/R model [3]. CK and ALP values were both noticeably low in group VI when compared to the other I/R groups, and a statistically significant difference was observed ($p<0.05$). Therefore, these enzymes may represent a diagnostic contribution in short-term I/R, in cases where anandamide is administrated in combination with CB1 receptor antagonist.

The results of this study suggest that the protective effect of endogenous anandamide against I/R injury is consequent to CB2 receptors in the small intestine; NO and prostaglandin, which are activated through the stimulation of CB2 receptors may be responsible for this protective effect.

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