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Local Administration of Adenosine and Adenosine A1 Receptor Agonist CPA Protects Against Intestinal Ischemia-Reperfusion Injury in Rats

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ABSTRACT

Objective: Adenosine and adenosine A1 receptor (A1AR) agonists have potential protective effects against reperfusion injury in variety of tissues. The purpose of the present study was to investigate possible effects of topical administration of adenosine and A1AR agonist on reperfusion-induced small intestinal injury in rat.

Materials and Methods: Rats were randomized to five groups each including six as following: sham-operated control; ischemia-reperfusion (I/R) control; adenosine + I/R; A1AR agonist 2-chloro-N^6-cyclopentyladenosine (CPA) + I/R; and A1AR antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) + adenosine + I/R. Following topical administration of the drugs into abdominal cavity for 5 min, intestinal I/R was established by clamping superior mesenteric artery (SMA) for 30 minutes followed by 180 min of reperfusion period. Afterwards terminal ileum samples were collected and immediately transferred to isolated organ bath for measuring contractile response to carbachol. Furthermore, additional tissue samples were harvested for measuring the levels of malondialdehyde (MDA) and reduced glutathione (GSH).

Results: I/R significantly increased lipid peroxidation while decreasing the GSH. Contractile responses were seriously reduced in I/R group compared to that of the sham control group. Pretreatment with adenosine or CPA not only decreased lipid peroxidation but also ameliorated contractile response and GSH levels remarkably. These beneficial effects were abolished by pretreatment with A1AR antagonist DPCPX.

Conclusion: Evidences we collected suggest that besides systemic administration, local application of adenosine and A1AR agonist CPA also attenuate ischemic intestinal injury via, at least, decreasing oxidative stress and enhancing antioxidant defense.

Key words: *Adenosine; adenosine A1 receptor; intestinal ischemia/reperfusion*

ÖZET

Adenozin ve Adenozin A1 Reseptör Agonisti CPA'nın Lokal Uygulanımı Sıçan İnce Barsak Hasarına Karşı Koruyucu Etkiler Göstermektedir

Amaç: Adenozin ve A1 adenozin reseptör (A1AR) agonistleri, çeşitli dokuların reperfüzyon hasarına karşı koruyucu etkilere sahiptir. Çalışmanın amacı, sıçan ince barsağının reperfüzyon harabiyetinde adenozin ve A1AR agonistinin lokal uygulanmasının etkilerini incelemekti.

Gereç ve Yöntemler: Sıçanlar herbiri altı hayvan içeren beş gruba rastgele olarak aşağıdaki gibi ayrıldı: sham kontrol; iskemi-reperfüzyon (I/R) kontrol; adenozin + I/R; A1AR agonisti 2-kloro-N^6-siklopentiladenozin (CPA) + I/R ve A1AR antagonisti 8-siklopentil-1,3-dipropilksantin (DPCPX) + adenozin + I/R. İlaçların abdominal boşluğa 5 dk boyunca lokal uygulanmasını takiben barsak I/R'u, süperiyor mezenter arterin 30 dk klempe edilmesi ve sonrasındaki 180 dk lık reperfüzyon dönemi ile sağlandı. Daha sonra terminal ileum örnekleri toplandı ve karbakole olan kasılma yanıtlarını ölçmek için hızlıca izole organ banyosuna alındı. Ayrıca malondialdehid (MDA) ve indirgenmiş glutatyon (GSH) düzeylerini ölçmek için de ek doku örnekleri alındı.

Bulgular: I/R, lipid peroksidasyonunu ileri düzeyde yükseltirken, indirgenmiş glutatyonu düşürdü. Sham kontrol grubuyla karşılaştırıldığında, kasılma yanıtları I/R grubunda ciddi düzeyde azaldı. Adenozin veya CPA ön tedavisi, sadece lipit peroksidasyonunu azaltmakla kalmadı aynı zamanda kasılma yanıtı ve GSH düzeyini de ileri derecede iyileştirdi. Bu yararlı etkilerin, A1AR anatgonisti DPCPX ön tedavisi ile ortadan kalktığı gözlendi.

Sonuç: Elde ettiğimiz kanıtlar, sistemik uygulanımının yanı sıra, adenozin ve A1AR agonisti CPA'nın lokal uygulanmasının da iskemik barsak harabiyetini, en azından oksidatif stresi azaltarak ve antioksidan savunmayı güçlendirerek iyileştirdiğini önermektedir.

Anahtar Sözcükler: *Adenozin, adenozin A1 reseptörü, barsak iskemi/reperfüzyonu*

Ischemia-reperfusion (I/R) injury of the intestine has been a serious problem in numerous situations such as small bowel transplantation, strangulated hernias, abdominal aortic aneurysm surgery, neonatal necrotizing enterocolitis, and cardiopulmonary bypass (1). Regardless underlying situation, intestinal I/R is related with decreased contractile activity, increased microvascular permeability, and dysfunction of mucosal barrier (2, 3). The development of intestinal I/R injury is a complex, multifactoriel pathophysiological process that involves the formation of oxygen free radicals (OFR) (2, 4-9), inflammatory cytokines, and neutrophil infiltration as well as activation of complement system at the site of injury (1-3, 6, 7, 10).

Increased cellular consumption of ATP, one of the events observed during ischemia, eventually leads to

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accumulation of adenosine; thereby, elevating extracellular level of adenosine, which is believed to provide cytoprotection to the ischemic tissue (11, 12). It has been shown that adenosine is the mediator that is responsible fot the early (13, 14) and late (11, 15, 16) phase of ischemic tolerance in myocardium. A number of studies in which adenosine receptor agonists and antagonists (15-18) as well as animals overexpressing or lacking A1AR (19, 20) have been employed, indicates that adenosine has potent anti-ischemic actions, minimizing I/R-related injury. It has been shown that administration of adenosine either before ischemia or during reperfusion attenuate myocardial injury (21, 22). Treatment with A1AR agonsit has been shown to induce beneficial effects not only in heart (15-18, 23) but also in kidney (20, 24) and brain (25, 26). One mechanism underlying the adenosine receptor-mediated protection involves activation of protein kinase C (PKC) in heart (11, 12, 27). PKC activation triggers increased opening of ATP-sensitive K^+ channels (11, 12, 28). Other effectors possibly contributing to cytoprotection by adenosine are mitogen activated protein kinases (MAPK) (23, 28-30), heat shock proteins (HSPs) (11, 12, 15), antioxidant enzymes (15, 31) and inducible nitric oxide synthase (iNOS) (11, 12). On the other hand, the physiological role of adenosine in the gastrointestinal tract is stil poorly understood, particularly with regard to colonic and ileal motor function. It has been reported that adenosine A1AR antagonists increase defecation in rats (32) and adenosine agonists can inhibit intstinal fluid secretion and peristalsis via adenosine $A2_B$ and A1 receptors, respectively (33). A previous study of our laboratory demonstrated that the systemic administration of adenosine and A1AR agonist CPA, prior to ischemia, attenuates reperfusion injury of the small intestine by decreasing oxidative stress, lowering neutrophil infiltration, and increasing GSH content (34).

Studies suggest an important anti-ischemic role for the A1ARs (16, 23, 27), A3ARs (18, 19) or $A2_A$ARs (35, 36) in heart. However, few data are available on the role of the different adenosine receptors in mediating cytoprotection in intestinal tissue. Adenosine may promote cytoprotection through activation of different types of receptors in different tissues. Moreover, there is no direct evidence showing effect of local administration of adenosine and A1AR activation on I/R-induced decreased contractility of intestinal smooth muscle. Thus, the present study was designed to determine the possible effects of adenosine and A1AR activation on tissue tolerance to prolonged ischemia by evaluating contractile response and levels of MDA (a product of lipid peroxidation) and GSH (an index of oxidative stress) in terminal ileum subjected to I/R.

MATERIAL AND METHODS

Thirty (30) Wistar adult male rats weighing 200-230 g were obtained from the Experimental Research Section of Zonguldak Karaelmas University. Animals were maintained in their cages at a constant room temperature using a 12 h: 12 h light/dark cycle and provided with commercially available rat chow and top water *ad libitum*. All experimental procedures described below were approved by the Ethical Committee of the University.

On day before the surgical procedures, the animals were fasted overnight but allowed ad libitum access to water. Animals were anesthetized with sodium thiopenthal (50 mg/kg) followed by a midline incision made into the peritoneal cavity. The small bowel was exteriorized gently to the left onto moist gauze; then, the SMA was carefully isolated and ligated by using a non-traumatic microvascular clamp. Following 30 min of occlusion time, the clamp was gently removed and the intestine inspected for proper reperfusion. During the surgical procedure, the animal was positioned under a heating lamp so that the body temperature was kept at constant level (i.e. 37 ºC). Animals were assigned to five groups: i) sham-operated control, subjected to laparatomy without performing the clamping of SMA; ii) I/R control, subjected to the clamping followed by reperfusion; iii) adenosine + I/R; iv) A1AR agonist CPA + I/R; and v) A1AR antagonist DPCPX + adenosine + I/R. In each treatment, 10 ml of warmed (37 ºC) saline solution containing either adenosine (10 nM) or A1AR agonist CPA (10 nM) was placed into abdominal cavity 5 min before inducing ischemia. To determine the adenosine receptor subtype involved in adenosine-induced intestinal protection, selective A1AR antagonist DPCPX (10 nM) was also administered topically 15 min before adenosine treatment. For the control groups, only saline solution was placed into the abdominal cavity. The solution with or without any treatment was let to stay in the abdominal cavity for 5 min followed by gentile soacking the solution with absorbent sterile gause. Dose regimen for adenosine and for specific agonist or antagonist was based on the literature (20, 37, 38).

Upon completing the 180 min of reperfusion period followed by exanguination of abdominal aorta, a segment (1 cm long) of terminal ileum 10 cm proximal to the ileocecal area was rapidly excised and transferred into a Petri dish containing Krebs solution (in mM: NaCl 118, NaHCO3 24.88, KH2PO4 1.18, KCl 4.7, MgSO4 1.16, CaCl2 2.52 and glucose 11.1). Then, the strip was suspended in a standard organ chamber which was continuously perfused with preoxygenated Krebs solution (pH 7,4), which was bubbled constantly with a mixture of %95 O_2 and %5 CO_2 and maintained at a temperature of 37° C. One end of the strips was tied to a fixed post and other attached to an isometric force transducer under a resting tension of 2 g. The isometric responses were recorded on the computer using MP 30 data acquisition and analysis system (Biopac Systems Inc., CA, USA). In organ bath, each strip was allowed to equilibrate for 1 h with intervening washings at every 15 min before adding any compound. Additional tissue samples were also taken from the same region and stored at - 40 ºC for biochemical measurements.

The contractile responses to various final doses of carbachol ranging from 10^{-9} M through 10^{-2} M were measured as it was pipetted into the organ bath in a cumulative fashion at equal intervals. The amplitude of contraction is expressed as grams per gram of tissue weigth. Each experiment was performed with a tissue sample taken from one animal.

Adenosine, carbachol, CPA, and DPCPX were purchased from Sigma (St. Louis, MO, USA). They were dissolved in double distilled water, except for CPA and DPCPX which were initially prepared in DMSO and then diluted in physiological saline. Adenosine, CPA, and DPCPX were prepared freshly just before usage. Carbachol was made up at different concentrations and kept frozen in aliquots. Compounds for Krebs solution were purchased from Merck (Merck KGaA, Darmstadt, Germany). All other reagents were obtained from Sigma.

Tissue MDA was measured to estimate the extent of lipid peroxidation in the tissues. Tissue samples were washed in ice-cold Krebs solution, blotted on absorbent paper and weight. Afterwards, each sample was minced followed by homogenizing with 10% TCA in ratio 1:10 w/v, using motor-driven homogenizer. Then, the tissue MDA levels were measured spectrophotometrically according to a method described by Casini et al (39) and expressed as nmol/g of tissue. The GSH content, expressed as μmol/g of tissue, was measured using a modified Ellman method (40).

Data are presented as the mean ± standard error (SE) of the mean. All statistical procedure was performed using SPSS statistical software (11.0, SPSS Inc., Chicago, IL, USA) package program. Kruskal-Wallis H test was applied for statistical comparison of groups, followed by analysis with Bonferroni-corrected Mann-Whitney test so as to determine the different groups. Probability values of 0.05 or less were considered statistically meaningful.

RESULTS

The addition of carbachol at concentrations from 10^{-9} M to 10^{-2} M into the organ bath resulted in a dose-dependent contractile effect on the terminal ileum segments from all groups, providing sigmoid curves with their maximum responses (E_{max}) (Figure 1). E_{max} value for carbachol was significantly lower in the I/R control group than in the sham control group (0.40±0.02 g/g tissue *vs* 1.33±0.28 g/g tissue, respectively). In other words, I/R significantly reduced the contractile response to carbachol (almost 3.5 fold). Comparing the E_{max} values, average contraction of ileum samples in I/R group was 30 % of that in sham control group, while those in adenosine-, CPA-, and DPCPX + adenosine-treated groups were approximately 107 %, 102 %, and 17 %, respectively. Compared to the average value in sham control group, that in DPCPX-pretreated adenosine group (0.23±0.06 g/g tissue) was significantly different (P<0.01). Statistical difference between the sham control and I/R control and DPCPX-pretreated groups appeared to be meaningful at 10^{-7} M and higher concentrations of carbachol. On the other hand, average E_{max} values in adenosine- (1.43±0.07 g/g tissue) and CPA- (1.36±0.14 g/g tissue) treated groups were statistically indifferent from that in sham-operated control group.

Approximately 1.5 fold increase in MDA content was measured in I/R control group, which is a significant difference (P<0.05) compared to that in sham control group (Table 1). Administration of either adenosine or CPA significantly reduced the elevated MDA content to the levels observed in sham control group. Thus, values of the both groups were not statistically different from that of the sham control group (P>0.05). On the other hand, comparing with I/R group, average MDA contents of the both groups were significantly different (P<0.05). In the case of DPCPX pretreatment before adenosine administration, the average MDA content was statistically indifferent from that measured in I/R control (P>0.05).

Amount of GSH measured in the I/R control group decreased approximately 58 % compared to that measured in the sham control (P < 0.05) (Table 1). Levels of tissue GSH were statistically indistinguishable between I/R control and DPCPX-pretreated adenosine groups (P > 0.05). However, both adenosine and CPA-treated groups significantly ameliorated the decreased amount of GSH. Mean GSH contents of these groups were not statistically different from that measured in sham control animals (P < 0.05).

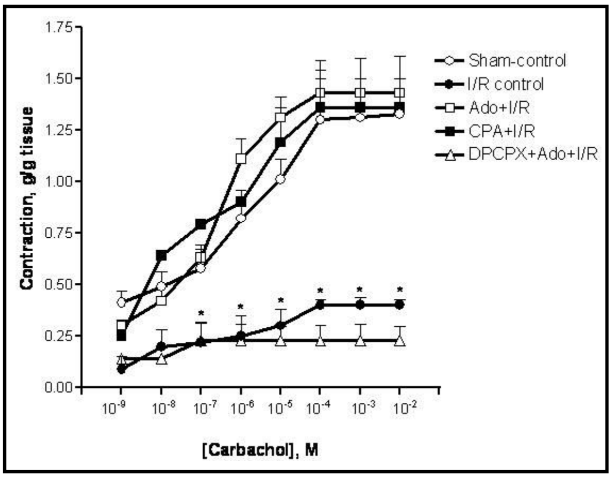

Figure 1. *Effects of Ado, CPA, and DPCPX + Ado on carbachol-induced contraction of ileum samples subjected to I/R. Dose-response curves of carbachol in longitudinal ileum muscle collected from sham control, I/R control, ado-treated+I/R, CPA-treated+I/R, and DPCPX-pretreated ado+I/R rats. Each data point is the mean±S.E.M. (n=6). *P<0.05 indicates statistical significance compared to sham control. (I/R: ischemia/reperfusion; Ado: adenosine; CPA: 2-chloro-N^6-cyclopentyladenosine; DPCPX: 8-cyclopentyl-1,3-dipropylxanthine).*

DISCUSSION

The major findings of our study are that: 1) I/R-induced decrease in contractility in response to carbachol was significantly restored by preischemic A1AR activation or adenosine administration; 2) The A1AR antagonist DPCPX blocked the injury-sparing effect of adenosine. Thus, pharmacological blockade of A1ARs worsened intestinal contractile response; 3) Adenosine and A1AR-mediated protection from I/R injury is associated with decreased oxidative stress and increased GSH level.

Ischemia-reperfusion (I/R) interrupts the exogenous electrical activity and contractile response of ileum (8-10). OFRs (1-3, 6, 8) and activated neutrophils (1-3, 10) have all related with the pathogenesis I/R and I/R-induced motor alterations. Intestinal I/R lays the foundation of an inflammatory response within the area of muscularis cells which ends up with the recruitment and extravasation of leukocytes into smoth muscle syncytium (2, 10, 34). Aiming to improve survival after acute mesenteric ischemia, a number of experimental studies have been carried out in order to test several pharmacological agents that might attenuate reperfusion injury of the intestinal mucosa (4, 5, 7, 9, 41, 42).

Both animal and human studies have identified adenosine as one of the most important triggers of ischemic tolerance. Unal et al demonstated that adenosine administration before ischemia is very efficient in inducing ischemic tolerance in rat (38). Our findings showed that local pretreatment with adenosine or A1AR agonist CPA restored I/R-reduced contractile response and GSH level as well as I/R-increased lipid peroxidation. Furthermore, the A1AR antagonist DPCPX blocked these cytoprotective effects of adenosine, consistent with the hypothesis that beneficial effect of adenosine is mediated via A1AR. These observations are in agreement with our previously published study in which systemic administration of adenosine and A1AR agonist was beneficial (34).

Preischemic activation of A1ARs has been shown to protect against I/R injury in many organs including heart (15-18, 23), kidney (24, 43) and brain (25, 26). Pharmacological blockade of A1ARs abolishes the acquisition of protection (15-18). The protective effect of A1AR activation involves the activation and translocation of PKC to sarcolemmal and to mitochondrial membranes. PKC activation leads to an increased opening of ATP sensitive K channels in heart (11, 12). Following A1AR stimulation, early activation of other kinases is required as well, such as tyrosine kinases, p38 MAPK (11, 23, 28, 30), ERK (29), and Akt (14). Associated with A1 agonist-induced ptotection, the enhanced content or activity of several proteins has been described. Among these proteins are HSP 27 (11) and Mn-SOD (15). It seems that these effector molecules differ among tissues. In small intestine, Davis et al demonstrated that pharmacological modulation of A1ARs was associated with reduced expression of P-selectin (37). In our study, I/R of small intestine increased MDA levels, indicating that OFRs produced as a result of I/R, thus inducing lipid peroxidation. Topical administration of adenosine and CPA appeared to be protective against I/R-induced reduction of contractility possibly via inhibiting lipid peroxidation, as confirmed by a reduction of MDA. An important observation is that activation of A1ARs in vitro protects against H_2O_2-induced cellular injury via signaling pathway associated with PKC (44). Similar observations are reported in studies modeling heart (45, 46) or kidney (44) in which A1ARs are involved in protection against H_2O_2-induced oxidative injury by modulation of the detrimental increases in intracellular calcium concentration and by means of activation of cardiomyocyte K channels after H_2O_2 exposure.

GSH is an endogenous antioxidant found in all animal cells. It reacts with free radicals and can provide protection from singlet oxygen, hydroxyl radical and superoxide anion (28). Several reports indicate that tissue injury, induced by various stimuli, coupled with glutathione depletion (9). In the present study, we showed that depletion of ileal tissue GSH was restored by adenosine and CPA pretreatments. These drugs leveled off GSH levels during reperfusion, an effect that may be related to PKC activation. Adenosine acts to induce the activation of antioxidant enzymes in vitro. This stimulatory action appears to involve PKC-mediated phosphorilation. Such mechanism could serve to decrease the levels of OFR, which would otherwise be harmful to the cell. This effect of adenosine was also evident in vivo, and may account for adenosine-induced reduction lipid peroxidation in cochlea (31). Although the present study did not examine other antioxidant enzymes and PKC, increased levels of GSH suggest a potential involvement of cytoprotective mechanism of adenosine and A1AR activation.

In this study, we demonstrated additional evidence that the local administration of both adenosine and A1AR agonist CPA also ameliorated the reduced-intestinal contractility induced by I/R. Furthermore, the findings suggest also that adenosine and/or A1AR agonist may exert this protective effect by decreasing oxidative stress and enhancing antioxidant defense. In conclusion, the cellular mechanism by which pharmacological activation of A1ARs attenuates intestinal injury, which may indicate the possibility of acute protective therapies for ischemia-related small bowel disease.

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Table 1. *Average values of MDA and GSH, measured in each group as mean ± S.E.M. (n = 6).*

GROUPS	MDA nmol/g tissue	GSH μmol/g tissue
Sham control	55,09 ± 2,58[a]	2,87 ± 0,28[a]
I/R control	84,54 ± 5,15[b]	1,21 ± 0,11[b]
Adenosine-treated group	54,87 ± 4,12[c]	2,33 ± 0,15[c]
CPA-treated group	50,49 ± 4,80[d]	1,98 ± 0,08[d]
DPCPX-Adenosine treated group	73,82 ± 0,70[e]	1,16 ± 0,03[e]

Analysis with Kruskal-Wallis H followed by Bonferroni-corrected Mann Whitney test. (a,b), (a,e), (b,c), (b,d) $p < 0.05$. (MDA: malondialdehid; GSH: reduced glutathione; SEM: standard error of the mean; I/R: ischemia/ reperfusion; CPA: 2-chloro-N^6-cyclopentyladenosine; DPCPX: 8-cyclopenty l-1,3-dipropylxanthine).

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