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Pretreatment with 5'-N-ethylcarboxamidoadenosine Provides Partial Improvement in Intestinal Ischemia-Reperfusion Injury of Rat

[Sıçan İnce Barsağının İskemi-Reperfüzyonunda 5'-N-etilkarboksiamidoadenozin Ön Tedavisi Kısmi İyileşme Sağlamaktadır]

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ABSTRACT

Objectives: Adenosine and adenosine A_1 receptor agonists exert protective effects against reperfusion injury in different tissues by mediating preconditioning. The aim of the present study was to examine the effects of adenosine A_1/A_2 receptor activation on reperfusion-induced small intestinal injury in rat.

Methods: Animals were randomized into four groups each including eight as following: sham control, ischemia-reperfusion control, 5'-N-ethylcarboxamidoadenosine (NECA) (non-selective A_1/A_2 agonist, 0.1 mg/kg, i.v.)-treated I/R, and theophylline (non-selective A_1/A_2 antagonist, 20 mg/kg, i.v.)-treated I/R groups. The treatments were administered 5 min before inducing ischemia in which superior mesenteric artery was clamped for 30 min followed by 180 min of reperfusion period. Myeloperoxidase, malondialdehyde, and reduced glutathione contents of terminal ileum samples were measured besides recording contractile responses to KCl, carbachol and substance P.

Results: Ischemic insult significantly increased neutrophil infiltration and lipid peroxidation while decreasing the reduced glutathione. Contractile responses were seriously reduced in I/R group compared to that of the sham control group. NECA pretreatment alleviated the tissue content of reduced glutathione remarkably besides providing partial amelioration of I/R-reduced contractile response, while theophylline pretreatment had no any protective effect.

Conclusion: We offer additional evidence that activation of A_1/A_2 adenosine receptors provides partial protection against ischemia-induced intestinal contractile dysfunction possibly through maintaining reduced glutathione content at physiological levels.

Key Words: Adenosine, intestine, ischemia reperfusion, NECA, rat, theophylline

ÖZET

Amaç: Adenozin ve adenozin A_1 reseptör agonistleri, çeşitli dokuların reperfüzyon harabiyetine karşı önkoşullanma yoluyla koruyucu etkiler göstermektedir. Çalışmanın amacı, sıçan ince bağırsağının reperfüzyon harabiyeti üzerine adenozin A_1/A_2 reseptör aktivasyonunun etkilerini incelemekti.

Yöntemler: Denekler herbiri rastgele sekiz hayvan içeren dört gruba ayrıldı: sham kontrol, iskemi-reperfüzyon kontrol, 5'-N-etilkarboksiamidoadenozin (NECA) (non-selektif A_1/A_2 agonisti, 0.1 mg/kg, i.v.) tedavili iskemi-reperfüzyon ve teofilin (non-selektif A_1/A_2 antagonisti, 20 mg/kg, i.v.) tedavili iskemi-reperfüzyon. Tedaviler iskemi yapılmadan 5 dk önce uygulandı. Süperiyor mezenter arter 30 dk klempe edildikten sonra reperfüzyon dönemi 180 dk sürdü. Terminal ileum segmentlerinin, KCl, karbakol ve substans P'ye olan kasılma yanıtlarının kaydedilmesinin yanısıra, dokuların miyeloperoksidaz, malondialdehit ve indirgenmiş glutatyon miktarları da ölçüldü.

Bulgular: İskemi, nötrofil infiltrasyonu ve lipid peroksidasyonunu ileri düzeyde yükseltirken aynı zamanda indirgenmiş glutatyonu düşürdü. Sham control grubuyla karşılaştırıldığında, kasılma yanıtları iskemi-reperfüzyon grubunda ciddi düzeyde azaldı. NECA ön tedavisi, doku indirgenmiş glutatyon miktarını ileri derecede düzeltti ve ayrıca iskemi-reperfüzyon sonucu azalmış kasılma yanıtını kısmen iyileştirdi. Teofilin ile ön tedavi ise herhangi koruyucu bir etki göstermedi.

Sonuç: Adenozin A_1/A_2 reseptör aktivasyonunun, iskemi sonucu gelişen bağırsak kasılma bozukluğuna karşı kısmi koruma sağladığını ve ayrıca bu korumanın, indirgenmiş glutatyon düzeyinin fizyolojik seviyede tutulması yolu ile muhtemelen gerçekleştiğine dair ek kanıt önermekteyiz.

Anahtar Kelimeler: Adenozin, bağırsak, iskemi reperfüzyon, NECA, sıçan, teofilin

Introduction

Ischemia-reperfusion (I/R) injury of the intestine plays a significant role in small intestine transplants, strangulated hernias, abdominal aortic aneurysm surgery, cardiopulmonary bypass, neonatal necrotizing enterocolitis, hemorrhagic, traumatic, or septic shock, and severe burns (1). It causes decreased contractile activity, increased microvascular permeability, and dysfunction of mucosal barrier (2,3). I/R injury of intestine is an intricate and multifactorial pathophysiological process in which the primary etiological agents are considered to be oxygen free radicals (OFRs) (4,5) derived mainly from xanthine oxide (2,3,6,7). Secondary pathological events are due to recruitment and activation of neutrophils in the intestine (6,10,11). Activated neutrophils that are accumulated in the intestinal mucosa augment ischemic injury by releasing OFRs and proteolitic enzymes (10,11). Consequently, inflammatory cytokines, complement system, and neutrophil infiltration contribute significantly to the development of the injury (1,2,6,8). I/R injury also results in disruption of exogenous electrical activity and contractile response of ileum (8,10). Reduced motility may cause ileus (5,12). These alterations in motility bring about bacterial overgrowth and translocations (10,12,13).

The purine nucleoside adenosine is among the major local regulators in physiological functioning of tissues. In the cases of abrupt disruption of energy supply (i.e. ischemia) and failure to meet cellular energy demand, its regulatory function becomes pronounced. Effects of adenosine are through its receptors, four of which, at least, have been cloned and characterized as A_1, A_{2A}, A_{2B}, and A_3 (14). The definitive physiological role(s) that adenosine plays in the gastrointestinal tract is still vague, particularly with regard to motor functions. It is reported that A_1 adenosine receptor (A_1AR) antagonists increase defecation in rats (14) and that A_1AR agonists can inhibit intestinal fluid secretion and peristalsis via adenosine A_{2B} and A_1 receptors, respectively (15). We already showed evidences that both adenosine and the A_1AR agonist 2-chloro-N6-cyclopentyladenosine (CPA) significantly ameliorate the damage exerted upon the ileum by I/R (16).

Anti-ischemic roles for the adenosine receptors in various organs are suggested in a number of studies. On the other hand, relatively less data are available on the role of the different adenosine receptors for mediating cytoprotection in intestinal tissue subjected to I/R. In our knowledge, there is no data directly providing evidence showing possible effects of A_1/A_2AR activation or inactivation on reduced contractility due to I/R. Therefore, the present study was designed to explore the possible effects of A_1/A_2AR activation on reperfusion injury of small intestine by evaluating contractile response and levels of malondialdehyde (MDA, a marker of lipid peroxidation), reduced glutathione (GSH, an endogenous antioxidant), and myeloperoxidase (MPO an index of neutrophil infiltration) in terminal ileum subjected to I/R.

Materials and Methods

Animals

All experimental procedures were approved by the Ethics Committee of the Medical School of Zonguldak Karaelmas University. Experiments were performed on 32 healthy adult male Wistar Albino rats (200-230 g) obtained from the Experimental Research Section of the University, where animals have been reared and maintained under standard conditions. Maximum care for humanely approach to animals was of primary purposes.

Chemicals

Theophylline, carbachol and substance P were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). NECA was obtained from MP Biochemicals (Solon, OH, USA). NECA and theophylline were dissolved in DMSO and prepared freshly just before usage. Carbachol and substance P were dissolved in double distilled water. They were made up at different concentrations and kept frozen in aliquots. All other reagents were obtained from Sigma.

Experimental groups, Induction of ischemia, Preparation of terminal ileum, and Ileal longitudinal muscle contractility

Animals were divided into 4 groups each including 8 animals: 1) Sham-operated control group, subjected to isolation of superior mesenteric artery (SMA) except for the occlusion; 2) I/R control group, subjected to the occlusion of SMA for 30 min followed by 180 min of reperfusion period; 3) NECA-treated group (0.1 mg/kg, 5 min prior to ischemia) + I/R; 4) Theophylline-treated group (20 mg/kg, 5 min prior to ischemia) + I/R. The route and volume for drug administration were the tail vein and 200 μl, respectively. Animals in the control groups were given saline in the same volume instead. Choice of dose regimen for the drugs was basically based on published studies in literature (19). Each animal was anesthetized with sodium thiopenthal (60 mg/kg, i.p.). The details of the surgical process were based on previously published studies (3,5)

Upon finishing up I/R, while still being unconcious, the animals were sacrificed by exsanguination of abdominal aorta. Strips of terminal ileum at 10 mm length were immediately removed at 10 cm away from the ileocecal junction. Further steps involved in sample preparation and organ bath were explained elsewhere (16). Tissue samples that were also obtained from the same region were frozen immediately and stored at – 40 °C for biochemical measurements.

Contractility of each ileum strip was tested first for high K^+ solution. The response to KCl was regarded as a refer-

ence response. Afterwards, the contractions in response to carbachol and substance P separately at various final concentrations ranging from 10^{-9} M to 10^{-2} M were recorded. Each compound was added into the organ bath in cumulative fashion. For evaluating the effects of ligand, agonist, and antagonist, the maximum response (E_{max}) and pD_2 values (e.g. the negative logarithm of the concentration for the half-maximal response, ED_{50}) were computed by using GraphPad Prism Software 3.02 (GraphPad Prism Inc., San Diego, CA, USA) (21). The pD_2 values, apparent agonist affinity constants, were calculated from each agonist concentration–response curve by linear regression of the linear median part of the sigmoid curve.

Measurement of tissue MDA and GSH

The tissue MDA levels (expressed as nmol/g of tissue) were measured spectrophotometrically based on a method described by Casini *et al.* (21). The GSH content (expressed as μmol/g of tissue) was measured using a modified Ellman method (22).

Measurement of tissue MPO activity

The degree of neutrophil accumulation in the intestinal tissue samples was measured by assaying MPO activity spectrophotometrically as described by Bradley *et al.* (23). One unit of MPO activity was defined as that degrading 1 μmol of peroxide per min at 25 °C. The activity was then normalized as unit per mg of tissue (U/mg).

Statistical analysis of results

Values for the contractility experiments were normalized for per g of tissue followed by expression of percentage of KCl response. Each data point represents mean ± 2S.E. For statistical evaluation, SPSS 11.0 statistical software package program was used (SPSS Inc., Chicago, IL, USA). 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

Ileal longitudinal muscle contractility

For sham control, NECA-treated, and theophylline-treated animals, mean contraction responses to 30 mM of KCl were measured as 0.59 ± 0.02 g; 0.40 ± 0.03 g; and 0.25 ± 0.03 g, respectively (Figure 1). I/R significantly reduced the contractile response (0.26 ± 0.04 g) in comparison with sham control group ($P < 0.0001$). NECA caused statistically significant contraction with respect to both I/R control group ($P = 0.028$) and sham control group ($P = 0.001$). On the other hand, the response recorded from theophylline treatment was statistically indifferent compared to that from I/R control ($P = 0.991$).

Carbachol caused dose-dependent contractions of samples in all groups, generating sigmoid curves with E_{max} and pD_2 values (Figure 2). E_{max} value for carbachol was significantly lower in the I/R control group than in the sham control group (166.49 ± 23.93 % *vs* 382.89 ± 46.07 %, respectively) (Table 1). In other words, I/R significantly reduced the contractile response to carbachol (almost 2.3 fold). Statistical difference between the sham control and other groups appeared to be meaningful at 10^{-6} M and higher concentrations of carbachol.

Comparing the E_{max} values, average contraction of ileum samples in I/R group was 43 % of that in sham control group, while those in NECA- and theophylline-treated groups were approximately 55 % and 38 %, respectively. Compared to average value in sham control group, those in other groups were significantly different ($P < 0.01$ for each one). In addition, E_{max} values in I/R, NECA-treated,

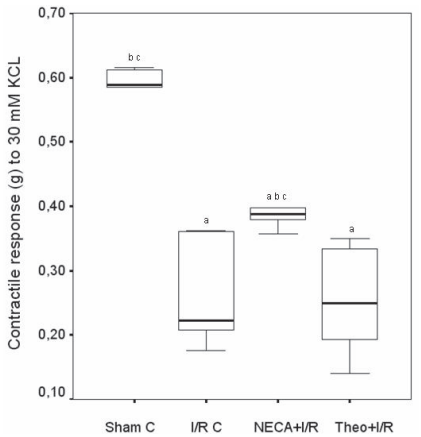

Figure 1. Effects of NECA and theophylline on KCl-induced contraction of ileum samples subjected to I/R. In response to 30 mM KCl, average contraction of longitudinal ileum muscle collected from sham control, I/R control, NECA-treated + I/R, and theophylline-treated + I/R rats (n = 8). aP < 0.05, bP < 0.05, and cP < 0.05 indicate statistical significance from sham control, I/R control, and theophylline-treated I/R groups, respectively.

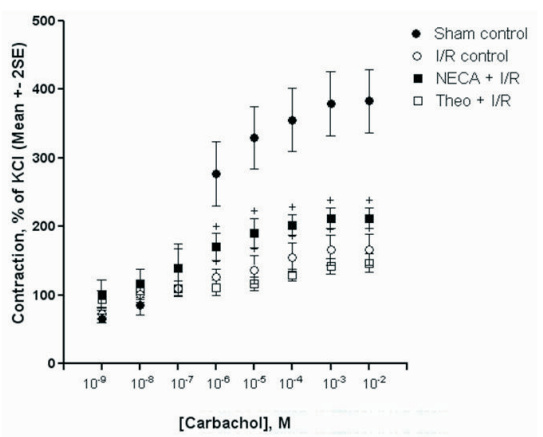

Figure 2. Effects of NECA and theophylline on carbachol-induced contraction of ileum samples subjected to I/R. Dose-response curves of carbachol in longitudinal ileum muscle collected from sham control, I/R control, NECA-treated + I/R, and theophylline-treated + I/R rats. Each data point is the mean ± 2S.E.M. (n = 8). +P < 0.05 indicates statistical significance compared to sham control.

Table 1. Emax (% of KCl) and pD2 values of longitudinal ileum muscle in response to carbachol and substance P.

	Sham Control	I/R Control	NECA	Theophylline
Carbachol				
Emax	382.89 ± 46.07*	166.49 ± 23.93+	211.69 ± 14.62+	146.78 ± 13.26+
pD2	6.36 ± 0.12	6.03 ± 0.35	6.37 ± 0.22	6.35 ± 0.34
Substance P				
Emax	345.66 ± 46.07*	134.56 ± 11.79+	243.51 ± 10.60*	107.77 ± 5.36+
pD2	6.15 ± 0.10	6.38 ± 0.32	5.99 ± 0.33	6.6 ± 0.33

Data shown as means ± S.E.M. (n = 8).
+P < 0.05, sham-operated control vs groups; *P < 0.05, I/R control vs groups.

and theophylline-treated groups were statistically indifferent. No statistically significant change was observed also in the corresponding pD_2 values in all groups (Table 1).

Responding to various concentrations of substance P, ileum samples contracted in a dose-dependent fashion in all groups, providing sigmoid curves with E_{max} and pD_2 values (Figure 3). The contraction was significantly and dose-dependently inhibited by induction of I/R. Statistically significant difference between sham and I/R control groups was observed at 10^{-6} M and over doses ($P < 0.05$). Reduced contractility due to I/R was significantly alleviated by NECA treatment at 10^{-4} M and higher doses ($P < 0.01$). At these concentrations, contractile responses of NECA-treated samples were significantly different from those of I/R control ($P < 0.05$) and theophylline-treated samples ($P < 0.01$), but indistinguishable from those of sham control samples ($P > 0.01$). Considering E_{max} values, average contractility in I/R group in response to substance P was reduced approximately 60 % compared to sham control animals. Regarding the pD_2 values, no statistically significant change was detected in all groups (Table 1).

MDA levels

Approximately 1.40 fold increase in MDA content was measured in I/R control group (80.75 ± 3.98 nmol/g tissue), a significant difference ($P < 0.001$) compared to that in sham control (57.61 ± 1.21 nmol/g tissue) (Figure 4). Administration of neither NECA nor theophylline had any effect on MDA content. Mean values of the both groups (86.96 ± 3.45 nmol/g tissue and 87.77 ± 0.55 nmol/g tissue, respectively) were significantly different from that of the sham control group ($P < 0.001$). On the other hand, comparing with I/R group, average MDA contents of both groups were statistically indifferent ($P > 0.05$).

GSH levels

Amount of GSH measured in the I/R control group (1.35 ± 0.2 μmol/g tissue) decreased approximately 54 % compared to that measured in the sham control (2.91 ± 0.6 μmol/g tissue) ($P < 0.001$) (Figure 5). Levels of tissue GSH were statistically indistinguishable between I/R control and theophylline-treated groups ($P > 0.05$). However, NECA significantly ameliorated the decreased amount of GSH. Mean GSH content was 2.11 ± 0.09 μmol/g tissue, which was significantly different from that measured in I/R control animals ($P < 0.01$).

MPO activity

MPO enzyme activities (as U/mg tissue) in sham control, I/R control, NECA-treated, and theophylline-treated groups averaged 9.23 ± 0.8, 17.25 ± 1.93, 14.20 ± 2.98, and 16.21 ± 0.88, respectively (Figure 6). I/R caused approximately 1.9 fold increase in MPO activity compared to the basal level (sham control) of the activity ($P < 0.0001$). Clearly, no statistical difference was observed in MPO activities of I/R control, NECA-treated, and theophylline-treated groups.

Discussion

The present study shows that intestinal I/R resulted in a significant decrease in ileal contractility in response to KCl, non-receptor-mediated induction, and both carbachol and substance P, receptor-mediated induction. Nevertheless, both types of induction provided statistically incomparable pD_2 values in all groups. Taken together with the finding that decreased contractile response of small intestinal smooth muscle was observed also in non-receptor-mediated induction, our data strongly suggest that intestinal I/R does not change ligand-receptor interaction but rather altering the regulation of post-receptor processes (i.e. excitation-contraction coupling) (3). Pretreatment with NECA rendered the improved-contractile responses to substance P at millimolar concentrations and to high K^+. The response to carbachol appeared to be statistically unmeaningful despite the higher values with NECA than those without any treatment. Treatment with theophylline, however, did have no effect on ischemia-reduced contractility whatsoever or on ischemia-induced oxidative stress. The present data

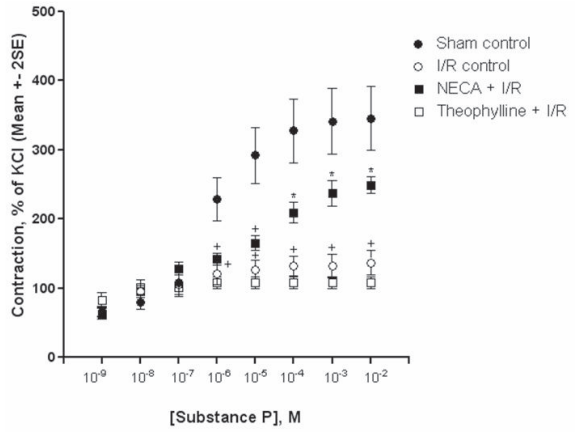

Figure 3. Effects of NECA and theophylline on substance P-induced contraction of ileum samples subjected to I/R. Dose-response curves of substance P in longitudinal ileum muscle collected from sham control, I/R control, NECA-treated + I/R, and theophylline-treated + I/R rats. Each data point is the mean ± 2S.E.M. (n = 8). +P < 0.05 and *P < 0.05 indicate statistical significance compared to sham control and I/R control groups, respectively.

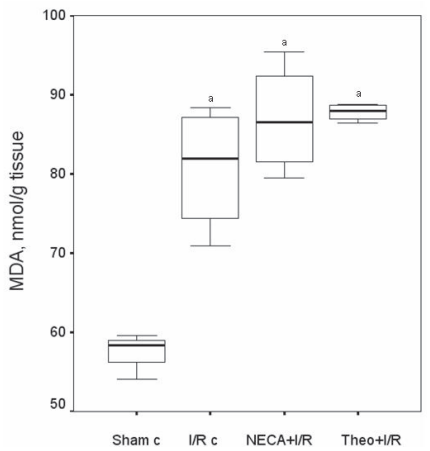

Figure 4. Effects of NECA and theophylline on lipid peroxidation of ileum tissue subjected to I/R. Average MDA content of ileum samples collected from sham control, I/R control, NECA-treated + I/R, and theophylline-treated + I/R rats (n = 8). aP < 0.05 indicates statistical significance compared to sham control.

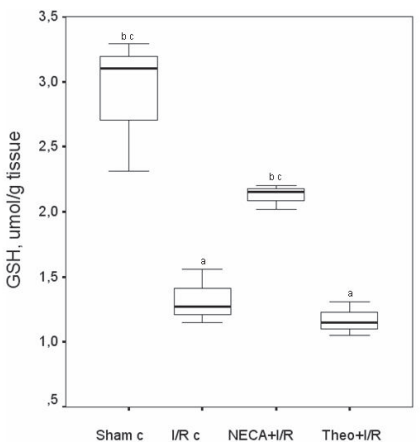

Figure 5. Effects of NECA and theophylline on GSH content of ileum tissue subjected to I/R. GSH content of ileum samples collected from sham control, I/R control, NECA-treated + I/R, and theophylline-treated + I/R rats (n = 8). aP < 0.05, bP < 0.05, and cP < 0.05 indicate statistical significance from sham control, I/R control, and theophylline-treated I/R groups, respectively.

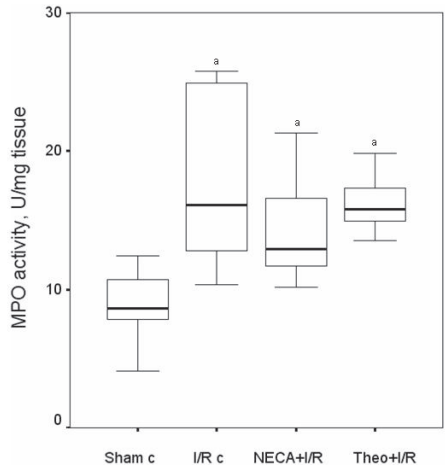

Figure 6. Effects of NECA and theophylline on neutrophil accumulation in ileum tissue subjected to I/R. Average MPO activity of ileum samples collected from sham control, I/R control, NECA-treated + I/R, and theophylline-treated + I/R rats (n = 8). aP < 0.05 indicates statistical significance compared to sham control.

demonstrated also that increased oxidative stress due to ischemic insult was lowered significantly by NECA as marked by restoring amount of GSH back to about control level. On the other hand, neither NECA nor theophylline had any effect on ischemia-induced increases in lipid peroxidation or neutrophil infiltration.

A substantial amount of evidence implicates that the pathogenesis of I/R and I/R-induced motor alterations have been related to both OFRs (1,2,6,10) and activated neutrophils (1,2,6,8). Reperfusion of ischemic tissue constitutes the major cause for the generation of harmfull OFRs (9). Moreover, an influx of neutropils during reperfusion triggers an intricate cascade of proinflammatory events associated. We have demonstrated that neither NECA nor theophylline prevented neutrophil infiltration into the reperfused-intestine. However, activation of A_1AR selectively is associated with decreased inflammation and MPO activity (16,29).

Some of the leading effector molecules that are involved in underlying mechanism responsible for adenosine receptor-mediated beneficial effects in heart are PKC (27), mitogen activated protein (MAP) kinases (25,26,27), heat shock proteins (HSPs) (14,15), inducible nitric oxide synthase (iNOS), and antioxidant enzymes (28). The similar beneficial effects are observed for such tissues as kidney (29) and brain (30) as well. The A_1/A_2AR agonist NECA decreases infarct size in heart and blocks formation of the mitochondrial permeability transition

pore by activating p70S6 kinase in cardiomyocytes (31). NECA limits infarction in heart also by activating phosphatidylinositol 3-kinase (PI3K), extracellular regulated kinase (ERK), and nitric oxide synthase (NOS) in signal pathway (32).

In the present study, intestinal I/R elevated the tissue MDA content, indicating enhanced generation of OFRs; therefore, inducing lipid peroxidation. NECA or theophylline appeared not to be significantly and completely protective against ischemia-reduced contractility. Unability of NECA and theophylline to inhibit lipid peroxidation and neutrophil infiltration may, in part, be underlying reasons for that. However, NECA ameliorated ischemia-reduced contractile response to 30 mM of KCl in addition to providing significant protection against ischemic reduction of ileal contractility in response to millimolar concentrations of substance P.

GSH, an endogenous antioxidant presented in all animal cells, reacts with OFRs, providing protection from damaging effects of singlet oxygen, hydroxyl radical and superoxide anion (18). We showed that depleted GSH content in the ischemic tissue was recovered remarkably by NECA administration. It is reasonable to implicate that activation of PKC may possibly be involved with this effect since adenosine is reported to induce the activation of antioxidant enzymes *in vitro* and since it is suggested that the stimulatory action of adenosine is likely involved in PKC-mediated phosphorilation. The same effect of adenosine is also evident in vivo, and may account for adenosine-induced reduction of lipid peroxidation in cochlea (28). It is reported that adenosine exerts these effects through mostly activating A_1 and possibly A_3 receptors. On the other hand, in the presence of oxidative stress, expression of A_2ARs, particularly A_{2A} subtype, decreases (28). Therefore, we speculate that the decrease in consumption of GSH that was observed with NECA treatment is mostly via activation of A_1ARs. We have already showed that pretreatment with the selective A_1AR agonist CPA prevents I/R-induced consumption of GSH in terminal ileum (16). While the current study has not quantified antioxidant enzymes or PKC, elevated level of GSH implicates a potential involvement of cytoprotective mechanism related to A_1/A_2 receptor activation. That is further supported by the observation that the non-selective A_1/A_2AR antagonist theophylline has had no any effect on ischemia-reduced GSH content of the tissue.

We have demonstrated that pretreatment with non-selective A_1/A_2AR agonist NECA partially ameliorated intestinal contractile dysfunction induced by I/R. On the other hand, pretreatment with non-selective A_1/A_2AR antagonist theophylline provided no any protective effect. Based on the data collected in the present study, we suggest that, activation of A_1/A_2 receptor types alleviates, in part, intestinal contractile dysfunction induced by I/R, possibly through maintaining GSH content at physiological levels.

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