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Attenuation of contractile dysfunction by atorvastatin after intestinal ischemia reperfusion injury in rats

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Abstract

Growing number of studies implicate that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or statins, have beneficial effects on ischemia/reperfusion injury that are unrelated to their cholesterol-lowering action. In the present study, we aimed to evaluate possible effects of atorvastatin on oxidative stress, neutrophil accumulation, and contractile response of terminal ileum segments in rats subjected to intestinal ischemia/reperfusion. Intestinal ischemia/reperfusion model was generated by clamping the superior mesenteric artery for 30 min followed by reperfusion for 3 h. Oral administration of atorvastatin at a dose of 10 mg/kg/day lasted 3 days just before induction of intestinal ischemia. At the end of reperfusion period, terminal ileum samples were removed to determine the concentrations of malondialdehyde, reduced glutathione, and myeloperoxidase. Samples were collected also to assess histopathological alterations and contractile response to agonists. Ischemia/reperfusion significantly decreased contractile responses, and this decrease was attenuated by atorvastatin. Pretreatment with atorvastatin caused remarkable decrease in both oxidative stress and neutrophil accumulation. Atorvastatin appeared to be restoring amount of reduced glutathione back to about control level. Furthermore, the pretreatment lowered mucosal damage at histopathological level.

Our results suggested that pretreatment with atorvastatin attenuated intestinal muscle dysfunction associated with ischemia/reperfusion. This remarkable effect of atorvastatin is accomplished at least by decreasing oxidative stress and neutrophil accumulation as well as preventing the depletion of reduced glutathione.

Keywords: Contractile function; Intestinal ischemia; Statin; Oxidative stress

1. Introduction

Intestinal ischemia–reperfusion injury is a critical condition arising from such dramatic insults as acute mesenteric ischemia, small bowel transplantation, abdominal aortic aneurysm, and hemorrhagic, traumatic or septic shock, or even severe burns (Naito et al., 2006; Mallick et al., 2004). Decreased contractile activity, increased microvascular permeability, and dysfunction of mucosal barrier are observed with intestinal ischemia/reperfusion (Carden and Granger, 2000). Various chemicals and cellular mediators have been implicated in the pathogenesis of intestinal ischemia/reperfusion, such as reactive oxygen species (Bielefeldt and Conklin, 1997; Ozacmak et al., 2005), cytokines, endotoxins, and neutrophils (Hierholzer et al., 1999). Following adhesive interactions among neutrophils and endothelial cells, neutrophil accumulation in the intestinal mucosa contributes to intestinal ischemia/reperfusion injury via production of reactive oxygen metabolites and proteases (Takagi et al., 2006). A substantial amount of evidence suggests that oxidative stress and excessive generation of reactive oxygen species are major players in ischemia/reperfusion (Bielefeldt and Conklin, 1997; Ozacmak et al., 2005).

A number of studies have shown that intestinal ischemia/reperfusion, induced by occlusion of the superior mesenteric artery followed by reperfusion, is involved in reduced gut motility that may result in ileus (Hierholzer et al., 1999; Ballabeni et al., 2002; Ozacmak et al., 2005). Various factors

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mediate motility changes such as reactive oxygen species (Hakguder et al., 2002; Ozacmak et al., 2005), inflammatory mediators, extravasated-leukocytes (Hierholzer et al., 1999), and altered nitric oxide (NO) metabolism (Takahashi et al., 2001).

Increasing number of evidence suggests that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, exert pleiotropic effects which are independent from their cholesterol-lowering action (Sanada et al., 2004; Endres and Laufs, 2004). One of these effects appears to be protection against ischemia/reperfusion injury. Several studies dealing with ischemia/reperfusion have shown that statins significantly reduce infarct size not only in heart (Birnbaum et al., 2005; Di Napoli et al., 2005) but also in brain (Hayashi et al., 2005; Laufs et al., 2002; Kawashima et al., 2003). Statins have been shown to elevate the expression of endothelial nitric oxide synthase (eNOS); hence enhancing the basal and stimulated production of NO and improving endothelium dependent vasorelaxation (Laufs et al., 2002; Shiga et al., 2005) besides promoting antiinflammatory processes (Arnaud et al., 2005; Pannu et al., 2005). In addition, statins upregulate eNOS via inhibition of geranylgeranylation of the small GTPase Rho. Rho-associated kinases are expressed in diverse types of smooth muscle as well as non-muscle cells, and it mediates Ca^{2+} sensitization (Fukata et al., 2001; Buyukafsar et al., 2006). They are involved in diverse cellular functions, including smooth muscle contraction, actin cytoskeleton organization, cell adhesion and motility, gene expression, and inflammation. Thus, inhibition of Rho kinases may contribute to some of the cholesterol-independent beneficial effects of statin therapy (Noma et al., 2006). It is implicated that their antiinflammatory effects may be associated with modulation of both adhesion molecule and cytokine production (Balduini et al., 2003; Pannu et al., 2005).

For the purpose of reducing intestinal ischemia/reperfusion injury, various treatment strategies have been demonstrated. Supplementation of NO or its precursor L-arginine aims at prevention of derangement of constitutive nitric oxide synthase (cNOS) and excessive superoxide ($O_2^{-\cdot}$) production (Kawata et al., 2001; Ward et al., 2000). Radical scavengers such as allopurinol (Gunel et al., 1998), superoxide dismutase (SOD) (Cuzzocrea et al., 2001), *N*-acetyl cysteine (Cuzzocrea et al., 1998) and antileukocyte therapy (Souza et al., 2001) have been reported to decrease intestinal ischemic damage. Statins seem to be promising for representing another approach to ischemia/reperfusion damage by decreasing inflammatory response and preserving of eNOS activity in several tissues including intestine (Naito et al., 2006). However, there is no direct evidence showing any effect of statin on ischemia/reperfusion induced reduction of intestinal smooth muscle contractility. Therefore, in the present study, possible protective effects of atorvastatin on ischemia/reperfusion induced intestinal tissue damage were examined by evaluation of contractile response as well as determination of tissue levels of malondialdehyde (MDA), myeloperoxidase (MPO) and reduced glutathione (GSH). Histopathological alterations were also examined in order to observe possible effect of atorvastatin administration. Choice of atorvastatin over other statins was basically because of both its usage in substantial amount of studies mentioned above and its relatively easy availability in our laboratory conditions.

2. Material and methods

2.1. Chemicals

Carbachol and substance P were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). They were dissolved in double distilled water. Different concentrations of carbachol and substance P were kept frozen in aliquots. Compounds, which were used for preparing Krebs solution, were purchased from Merck (Merck KGaA, Darmstadt, Germany). All other reagents, including trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxy toluene (BHT), dithiobisnitrobenzoate (DTNB), hexadecyltrimethylammonium bromide (HETAB), and *o*-dianisidine were obtained from Sigma.

2.2. Animals

Following Ethical Committee approval, twenty-four (24) adult male Wistar rats, weighing 230–250 g, were obtained from the Experimental Research Section of Zonguldak Karaelmas University, where animals have been reared and maintained under standard conditions, such as stable room temperature (23±2 °C), a 12 h light: 12 h dark cycle, and feeding with commercial rat chow and tap water *ad libitum*. On the day before the surgical procedures, the animals were fasted overnight but allowed freely to access water. Experimental manipulations and surgical operations of the study were approved by the Animal Ethical Committee of the University. Maximum care for humanely approach to animals was of primary purposes.

2.3. Induction of ischemia

Rats were anaesthetized with sodium thiopental (60 mg/kg, i.p.) followed by conducting laparotomy through a midline incision into the peritoneal cavity. After the small bowel was exteriorized gently to the left on to moist gauze, animals were subjected to 30 min of ischemia by occlusion of the superior mesenteric artery, using a microvascular clamp. Intestinal ischemia was confirmed by observing loss of pulsation of the mesenteric artery and its branches as well as paleness of the jejunum and ileum. Afterwards the intestines were returned to the abdomen which was then closed with two small clamps. At the end of 30 min of ischemia, the clamp was gently removed to allow reperfusion of the blood flow which was confirmed by observing the pulsation of the artery and its branches on the intestine. Body temperature was maintained at 37 °C by a heating lamp during the whole procedure of ischemia/reperfusion. In the experimental protocol, animals were divided into 4 groups each including 6 animals: 1) Sham operated control group: the rats underwent laparotomy and isolation of superior mesenteric artery with the exception of the occlusion of superior mesenteric artery; 2) Atorvastatin

treated sham group: rats subjected to sham operation were pretreated with atorvastatin for 3 days beforehand; 3) Ischemia/reperfusion control group: the superior mesenteric artery was occluded for 30 min followed by 3 h of reperfusion period; 4) Atorvastatin treated ischemia/reperfusion group: rats subjected to ischemia/reperfusion were pretreated with atorvastatin for 3 days before inducing ischemia.

Atorvastatin (Lipitor 20 mg, Pfizer Ilaclari Ltd. Sti) was administered daily at a dose of 10 mg/kg orally for 3 days before superior mesenteric artery occlusion. Each pill was carefully crushed followed by appropriate weighing of the powder for each animal. Introduction of the drug orally was carried out by mixing it with peanut butter, while animals in control groups received only peanut butter. All animals showed remarkable adjustment for eating the newly introduced food in the training period. Choice of dose regimen and timing for atorvastatin was basically based on published studies in literature (Yrjanheikki et al., 2005; Atar et al., 2006).

2.4. Preparation of terminal ileum

Upon finishing up the ischemia/reperfusion period, while still being unconscious, the animals were sacrificed by exsanguination of abdominal aorta. Strips of terminal ileum at 10 mm length were immediately removed 10 cm oral from the ileocecal junction and transferred into a Petri dish containing Krebs solution (in mM: NaCl 118, $NaHCO_3$ 24.88, KH_2PO_4 1.18, KCl 4.7, $MgSO_4$ 1.16, $CaCl_2$ 2.52 and glucose 11.1). Then, the strip was longitudinally suspended in a standard organ chamber which was continuously perfused with 20 ml of preoxygenated Krebs solution (pH 7.4), which was bubbled constantly with a mixture of 95% O_2 and 5% CO_2 gas and maintained at a temperature of 37 °C. One end of the strip was tied to a fixed post and the other attached to an isometric force transducer under a resting tension of 2 g. Isometric responses were monitored by external force displacement transducer (FDA-10A, Commat Iletisim Co., Ankara, Turkey) and recorded on the computer using MP 30 software (Biopac Systems Inc., Santa Barbara, CA, USA). In the organ bath, each strip was allowed to equilibrate for 1 h with intervening washings every 15 min before adding any compound. Tissue samples that were also obtained from small intestine 10 cm proximal to the ileocecal area were frozen immediately and stored at −40 °C for biochemical measurements. Some other samples that were taken from the same region were immediately placed in 37% formalin for histopathological examination.

2.5. Ileal longitudinal muscle contractility

At the beginning of each experiment, which was aiming to observe dose-response relationship, KCl was added to the organ chamber as its final concentration would be 30 mM. For the preparation of high K^+ solutions, NaCl was exchanged for equimolar amount of KCl so as to maintain the physiological osmolarity of the Krebs solution. The contraction recorded in response to KCl was considered as a reference response. Afterwards, the contractions in response to carbachol or substance P at various final concentrations ranging from 10^{-9} M to 10^{-2} M were recorded as these compounds were pipetted into the organ bath in a cumulative fashion at equal intervals. At the end of the experiment, the response to 30 mM of KCl was measured again to confirm and evaluate the degree of tissue viability. The amplitude of all contractions is then normalized for each g of tissue and expressed as a percentage of the initial KCl-reference response. Each experiment was performed with a tissue sample taken from one animal. For the purpose of evaluating the effects of agonist, the maximum response (E_{max}) and pD_2 values (e.g. the negative logarithm of the concentration for the half-maximal response, ED50) were computed by using GraphPad Prism Software 3.02 (GraphPad Prism Inc., San Diego, CA, USA). 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 and taken as a measure of the sensitivity of the tissues to each agonist.

2.6. MDA determination

Intestinal lipid peroxide levels were measured by a method described by Casini et al. (1989). Briefly, by using a motor-driven pestle, tissue samples were homogenized in ice-cold TCA by adding 10 ml of 10% TCA per g of tissue. After centrifugation, 750 μl supernatant containing 10 μl of 1% BHT was added to equal volume of 0.67% TBA and heated to 100 °C for 15 min. The absorbance was measured spectrophotometrically at 535 nm (Smart Spectro, LaMotte Co., Chestertown, MD, USA).

2.7. GSH determination

GSH contents of the samples were measured by a modified Ellman method (Aykac et al., 1985). To the 0.5 ml of supernatant obtained by using the same homogenization procedure as described above, 2 ml 0.3 M Na_2HPO_4 solution was added. A 0.2 ml solution of DTNB was added into the mixture, and the absorbance at 412 nm was measured immediately after vortexing.

2.8. Measurement of tissue MPO activity

The degree of neutrophil accumulation in the intestinal tissue samples was measured by assaying MPO activity as described by Bradley et al. (1982). Briefly, upon thawing, each sample was very finely minced with surgical blade in a Petri dish containing 50 mM potassium phosphate buffer (PB, pH 6.0) at a volume 20 times the tissue weight (e.g. 1 ml) followed by homogenizing for 5 min in ice-cold PB by means of motor-driven homogenizer. The homogenate was centrifuged at 40,000 *g* for 15 min at 4 °C. Homogenized tissue pellet was suspended in 50 mM of PB containing 0.05% HETAB; and then, homogenized again. Following three freeze and thaw cycles with sonication (Bandelin Sonopuls HD2070, Bandelin Electronic GmbH and CO.KG, Berlin, Germany) between cycles, the samples were centrifuged at 40,000 *g* for 10 min. Aliquots of supernatant (0.1 ml) were added to 2.9 ml of reaction mixture containing 0.167 mg/ml of *o*-dianisidine, and 20 mM H_2O_2 solution, which were prepared in 50 mM of PB. Immediately after adding the aliquot to the mixture,

the change in absorbance at 460 nm was measured for 5 min. 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).

2.9. Histopathological evaluation

Segments of ileum were fixed in 37% formalin and processed in acetone, alcohol, and formalin solution series for a total of 14 h. The specimens were then embedded in paraffin. Sections, which were 4 μm thick, were cut and stained with hematoxylin–eosin (H&E) for morphological analysis. Histopathological examination of reperfused intestinal tissue was based on a staging method described by Hierholzer et al. (1999); and, the evaluation was graded from 0 to 4. In grade 0, no specific pathological changes are observed: Normal gut wall architecture, including villi, crypts, lamina propria and muscularis externa. In grade 1, mild mucosal damage is assessed: Denudation of villi epithelium, otherwise normal structure. In grade 2, moderate damage occurs: Loss of villus length and epithelial sloughing with evidence of congestion, hemorrhage, and inflammation in the mucosa, but no change in submucosa or muscularis externa. In grade 3, an extensive damage is observed: Loss of a large number of villi including denudation, sloughing, and the presence of granulomatous tissue with the damage localized to submucosa and muscularis. In grade 4, there is severe damage and necrosis: Inflammation and necrosis in areas throughout the thickness of the intestinal wall.

2.10. Statistical analysis

Values for the experiments dealing with contractility were normalized for per g of tissue followed by expression of percentage of KCl response. Each data point represents mean± S.E.M. For statistical evaluation, SPSS 11.0 statistical software package program was used (SPSS Inc., Chicago, IL, USA). Nonparametric tests were performed since each group consisted of six replicate samples. Thus, using Kruskal–Wallis variance analysis (ANOVA), all groups were compared in terms of existence of heterogeneity. Once statistically meaningful difference was determined, individual groups were compared with each other as paired data, by employing Tukey test. P values of less than 0.05 were considered significant.

3. Results

3.1. Ileal longitudinal muscle contractility

Of samples harvested from sham operated and atorvastatin-pretreated ischemia/reperfusion animals, mean contraction responses to 30 mM KCl were measured as 0.60±0.02 g and 0.59±0.08 g, respectively, which were statistically indistinguishable (P=0.997) (Fig. 1). In ischemia/reperfusion control group, however, contractile response (0.27±0.04 g) was significantly reduced when compared to that in sham operated control group (P=0.001). Contractile response of atorvastatin-treated sham control was statistically indifferent from that of sham control (P=0.864).

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 E_{max} and pD_2 values (Fig. 2). E_{max} value for carbachol was significantly lower in the ischemia/reperfusion control group than in the sham operated control group (161.40±7.57% vs. 365.30±8.50%, respectively.) (Table 1). In other words, contraction in response to carbachol was significantly and dose-dependently reduced by induction of ischemia/reperfusion as indicated with right-shifted curve. Statistical difference between the groups appeared to be meaningful at 10^{-6} M dose of carbachol (P=0.007). The ischemia/reperfusion induced reduction in contractility was significantly ameliorated by pretreatment with atorvastatin. Restoration of contractions with atorvastatin therapy started to be statistically significant at micromolar doses of carbachol when compared to ischemia/reperfusion control group (P=0.049; P=0.001; P=0.002; and P=0.001; at 10^{-5} M; 10^{-4} M; 10^{-3} M; and10^{-2} M, respectively). As seen on Fig. 2, treatment of sham animals with atorvastatin has provided the same contractile response curve with similar E_{max} value as sham alone group does.

Comparing E_{max} values, average contraction of ileum samples in ischemia/reperfusion group was only 44% of that in sham operated control group (P=0.001). Average contraction in sham operated control group was statistically incomparable with that in atorvastatin-pretreated group (P=0.674) (Table 1). On the other hand, no statistically significant change was detected in the corresponding pD_2 values in all groups (Table 1).

In response to various concentrations of substance P ranging from 10^{-9} M to 10^{-2} M, terminal ileum samples contracted in a dose-dependent fashion in all groups, rendering sigmoid curves with E_{max} and pD_2 values (Fig. 3). The contractile response induced by substance P was significantly and dose-dependently

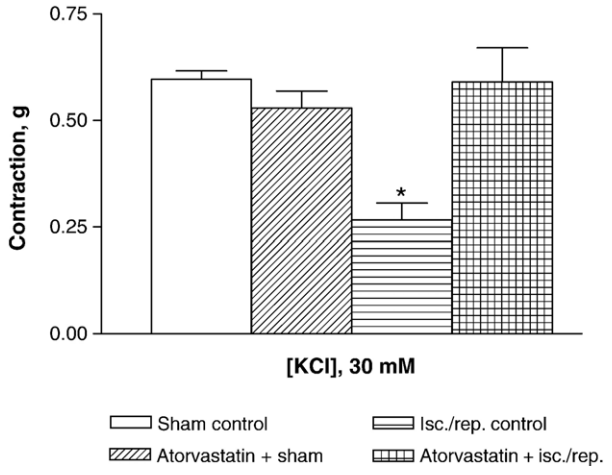

Fig. 1. Effect of atorvastatin on KCl-induced contractility of ileum samples during intestinal ischemia/reperfusion. In response to 30 mM KCl, mean contraction of longitudinal ileum muscle isolated from sham operated control, atorvastatin-pretreated sham, ischemia/reperfusion control, and atorvastatin-pretreated ischemia/reperfusion rats. Data are expressed as means±S.E.M. (n=6). * P<0.05 indicates statistical significance from sham operated control group.

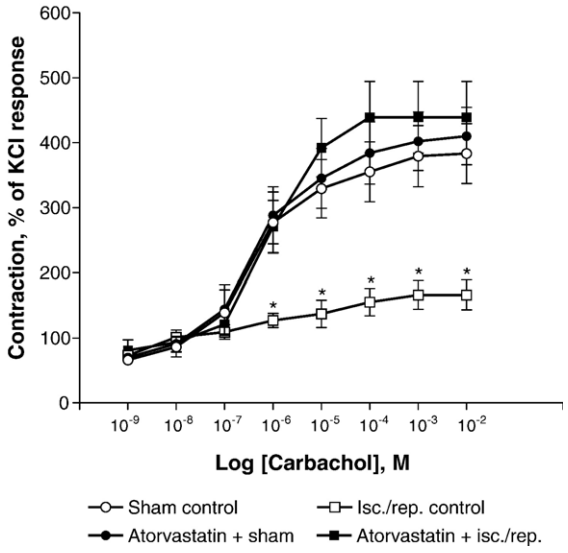

Fig. 2. Effect of atorvastatin on carbachol-induced contractility of ileum samples during intestinal ischemia/reperfusion. Concentration-response curves of carbachol in longitudinal ileum muscle isolated from sham operated control, atorvastatin-pretreated sham, ischemia/reperfusion control, and atorvastatin-pretreated ischemia/reperfusion groups. Each data point is the mean±S.E.M. of 6 experiments. * $P<0.05$ indicates statistical difference vs. sham operated control.

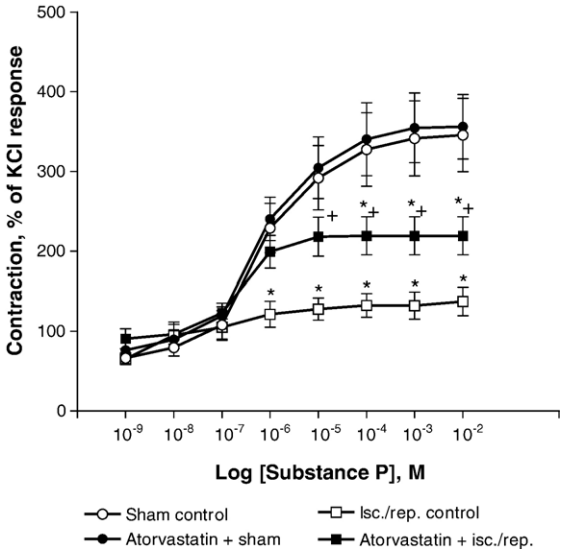

Fig. 3. Effect of atorvastatin on substance P-induced contractility of ileum samples during intestinal ischemia/reperfusion. Concentration-response curves of substance P in longitudinal ileum muscle isolated from sham operated control, atorvastatin-pretreated sham, ischemia/reperfusion control, and atorvastatin-pretreated ischemia/reperfusion groups. Each data point is the mean± S.E.M. of 6 experiments. * $P<0.05$ and $^{+}P<0.05$ indicate statistically meaningful differences when compared to sham operated control group and ischemia/reperfusion control group, respectively.

inhibited by induction of ischemia/reperfusion. Statistical difference between sham operated control and ischemia/reperfusion control was significant at 10^{-6} M ($P=0.004$) and over doses of substance P ($P<0.01$). Reduced contractility due to ischemia/reperfusion was significantly ameliorated by atorvastatin pretreatment which was statistically meaningful at 10^{-5} M of substance P ($P=0.001$). Similarly, significant difference existed between ischemia/reperfusion control and atorvastatin-pretreated groups in response to 10^{-4} M ($P=0.002$), 10^{-3} M ($P=0.002$), and 10^{-2} M ($P=0.004$) of substance P as well. Considering E_{max} values, average contractility in ischemia/reperfusion group in response to substance P was reduced approximately 61%, compared to sham control animals ($P=0.0001$). Regarding the corresponding pD_2 values, no statistically significant change was detected in all groups (Table 1). Additionally, pretreating sham animals with atorvastatin caused similar dose-response curve to sham operated group in respect to E_{max} and pD_2 values.

Table 1
E_{max} and pD_2 values of longitudinal ileum muscle in response to carbachol and substance P

	Sham control	Atorvastatin+ sham	Ischemia/ reperfusion control	Atorvastatin+ ischemia/ reperfusion
Carbachol				
E_{max}	365.30±8.50	410.00±44.22[b]	161.40±7.57[a]	436.40±4.27[b]
pD_2	6.36±0.12	6.21±0.18	6.03±0.35	6.03±0.04
Substance P				
E_{max}	333.2±7.04	356.21±40.55[b]	129.8±4.16[a]	220.3±1.53[b]
pD_2	6.15±0.10	6.29±0.21	6.38±0.32	6.60±0.05

Each value is the mean±S.E.M. of E_{max} (maximum contraction response, as % of KCl response) or pD_2 (the negative logarithm of the concentration for the half-maximal response) obtained from 6 experiments. "a" and "b" $P<0.05$, statistically different when compared to sham operated control and ischemia/reperfusion control, respectively.

3.2. MDA levels

Average MDA content of intestinal samples from sham operated animals was 54.18±1.63 nmol/g tissue, while that from

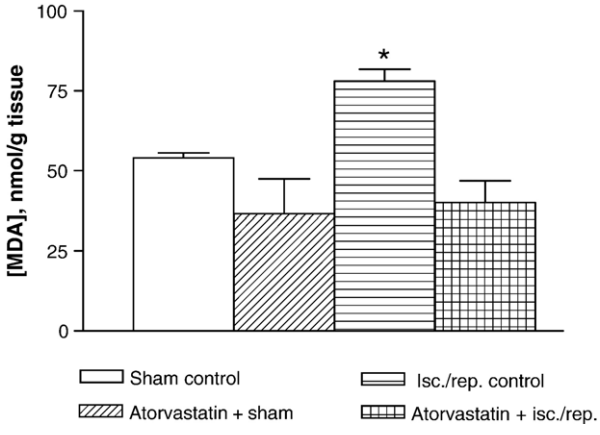

Fig. 4. Effect of atorvastatin on lipid peroxidation during intestinal ischemia/reperfusion. Average malondialdehyde (MDA) content of ileum samples from sham operated control, atorvastatin-pretreated sham, ischemia/reperfusion control, and atorvastatin-pretreated ischemia/reperfusion groups. Data are expressed as mean±S.E.M. ($n=6$). Statistically significant difference is denoted as "*" ($P<0.05$), compared to sham operated control group.

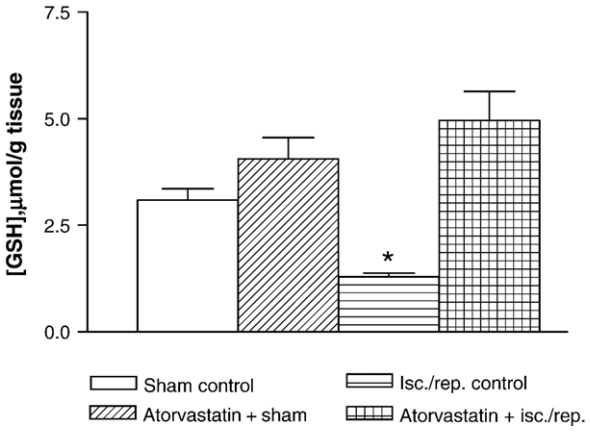

Fig. 5. Effect of atorvastatin on reduced glutathione levels during intestinal ischemia/reperfusion. Average reduced glutathione (GSH) content of ileum samples from sham operated control, atorvastatin-pretreated sham, ischemia/reperfusion control, and atorvastatin-pretreated ischemia/reperfusion groups. Data are expressed as mean±S.E.M. ($n=6$). Statistically significant difference is denoted as "*" ($P<0.05$), compared to sham operated control group.

ischemia/reperfusion control rats was 78.27±3.80 nmol/g tissue (Fig. 4). Ischemia/reperfusion caused approximately 1.45 fold increase in MDA content of the tissue, a significant difference compared to sham control animals ($P=0.024$). Administration of atorvastatin prior to the induction of ischemia significantly reduced the elevated MDA content to the levels observed in sham control group. Mean value of the pretreated group (40.19± 6.85 nmol/g tissue) was significantly different from that of the ischemia/reperfusion control group ($P=0.001$). On the other hand, average MDA contents of tissues from both sham control and atorvastatin-pretreated (both sham and ischemia/reperfusion) animals were statistically indifferent from each other ($P=0.175$).

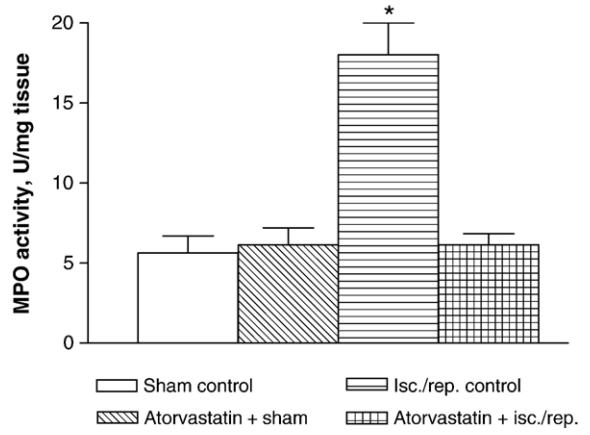

Fig. 6. Effect of atorvastatin on neutrophil accumulation in ileum segments during intestinal ischemia/reperfusion. Average myeloperoxidase (MPO) activity of ileum samples collected from sham operated control, atorvastatin-pretreated sham, ischemia/reperfusion control, and atorvastatin-pretreated ischemia/reperfusion groups. Data are presented as mean±S.E.M. of 6 to 8 animals in each group. * $P<0.05$ denotes statistically significant difference when compared to sham operated control group.

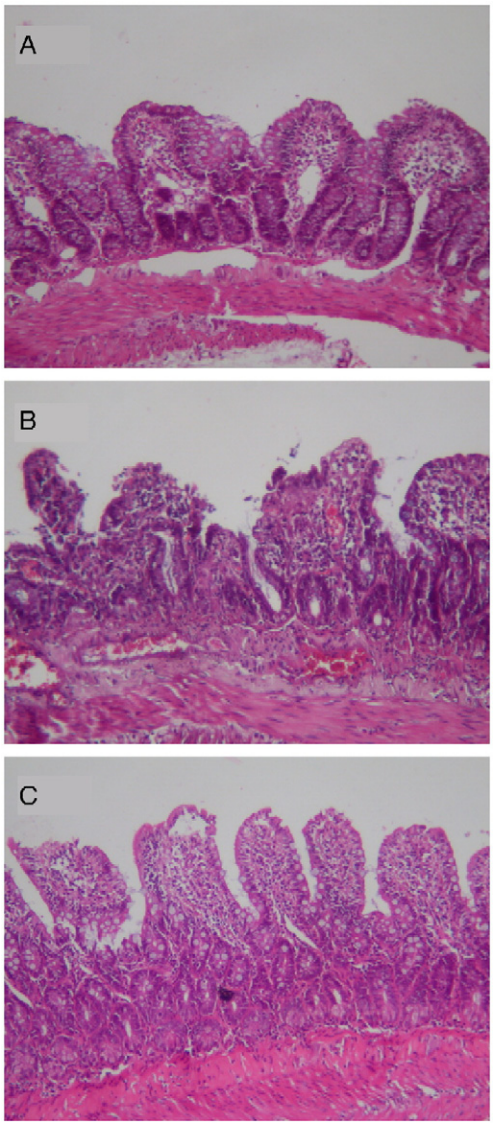

Fig. 7. Effect of atorvastatin on histological appearance of small intestine subjected to ischemia/reperfusion. Light micrographs of rat intestinal tissue: (A) Sham operated control group; normal mucosal architecture (grade 0); (B) ischemia/reperfusion group; the most extensive morphological changes detected (grade 2); (C) Atorvastatin-pretreated group+ischemia/reperfusion; the pretreatment ameliorates the histopathological alterations observed after ischemia/reperfusion (grade 0). (H&E stain ×200).

3.3. GSH levels

As demonstrated on Fig. 5, amount of GSH measured in the tissues subjected to ischemia/reperfusion (1.30±0.09 µmol/g tissue) decreased approximately 60% compared to that measured in the tissues from sham operated group (3.20±0.27 µmol/g tissue) ($P<0.03$). Pretreatment with atorvastatin significantly ameliorated the decreased amount of GSH. Mean GSH content of the pretreated group was 4.96±0.68 µmol/g tissue, which was significantly different from that measured in ischemia/reperfusion control animals ($P=0.001$). GSH contents measured in samples from sham operated control animals and atorvastatin-pretreated (both sham and ischemia/reperfusion) animals were statistically indistinguishable ($P=0.074$).

3.4. MPO activity

MPO enzyme activities in the terminal ileum samples from animals subjected to sham operation, ischemia/reperfusion, and atorvastatin pretreatment + ischemia/reperfusion, averaged 5.63 ± 1.06 U/mg tissue, 18.02 ± 1.97 U/mg tissue, and 6.14 ± 0.69 U/mg tissue, respectively (Fig. 6). The enzyme activity in atorvastatin-treated sham group was basically the same as that in sham alone group.

Ischemia/reperfusion caused 3.2 fold increase in MPO activity compared to the basal level of the activity ($P < 0.001$), which was measured in tissues of sham control animals (Fig. 6). In the case of atorvastatin-pretreatment, average MPO activity was statistically indifferent from that in sham operated control animals ($P = 0.973$). Accordingly, in terms of average MPO activities, atorvastatin-pretreated group was significantly different from ischemia/reperfusion control group ($P = 0.001$) as shown by 2.9 fold decrease in the activity of the pretreated group.

3.5. Histopathological findings

Based on histopathological analysis of 5 sections for each group, the most extensive changes in morphology were evident in ischemia/reperfusion control group (Fig. 7B). Mucosal denudation, edema, and inflammation were clearly observed on some mucosal and submucosal areas; thus, noticed as grade 2. In sham operated control group, no any pathological change was detected as demonstrated on picture A, grading as 0 with normal structure of epithelium. The analysis also showed that the pretreatment with atorvastatin restored typical alterations in morphology remarkably as depicted on picture C, grading as 0. Sections obtained from atorvastatin-pretreated sham group were observed with normal morphological appearance identical to those from sham operated control group (data not shown).

4. Discussion

The present study demonstrated that intestinal ischemia/reperfusion caused a significant decrease in ileal contractility in response to both carbachol and substance P, receptor-mediated induction, and KCl, non-receptor-mediated induction. That the pD_2 values in all groups were found to be statistically unchanged, however, suggests that ischemia/reperfusion does not alter ligand–receptor interaction. Therefore, the reduced E_{max} values in ischemia/reperfusion may be dependent partly on change in the regulation of postreceptor processes. Decreased contractile response observed also in non-receptor-mediated induction supports the possibility that ischemia/reperfusion may not alter ligand–receptor interaction but rather changing the regulation of postreceptor processes (i.e. excitation-contraction coupling) (Ozacmak et al., 2005). Pretreatment with atorvastatin appeared to be improving the reduced contractile responses remarkably. Potential influence of atorvastatin is shown to be multifold including microcirculatory, antiinflammatory and antioxidant effects on different systems. Atorvastatin pretreatment lowered the MDA content of intestinal tissue to those observed in sham control animals. The related data demonstrated preventive effect of atorvastatin against ischemia-induced oxidative injury in small intestine as maintaining GSH content on control levels. Atorvastatin pretreatment also lowered MPO activity to levels observed in sham control animals; exerting antiinflammatory effect. In addition, histopathological analysis indicated that intestinal ischemia/reperfusion induced severe injury in intestine. It is noteworthy to express that the efficacy of atorvastatin administration was also evident in sections of terminal ileum samples.

In general, one of the initial events observed in ischemia/reperfusion is the production of reactive oxygen species, which seems to be in charge of the generation of chemotactic activity for neutrophils. Intestinal ischemia/reperfusion causes such dramatic damages as disruption of the exogenous electrical activity and contractile response of ileum (Takahashi et al., 2001; Ballabeni et al., 2002). Intestinal ischemia/reperfusion injury triggers development of inflammatory response within muscularis cells of the intestinal wall, which results in recruitment and extravasation of leukocytes into the smooth muscle syncytium; thus, contributing to muscle dysfunction observed after ischemia/reperfusion (Hierholzer et al., 1999). In hypoxic conditions (i.e. ischemia), energy-rich phosphates are depleted that initially leads to either a reversible decrease or complete cessation of cell functions such as contractility. A considerable amount of study implicates that reactive oxygen species are related with the pathogenesis of intestinal ischemia/reperfusion-related motor alterations (Hakguder et al., 2002; Ozacmak et al., 2005). In order to observe pathophysiological alterations due to ischemia/reperfusion injury without completely losing agonist-induced dysmotility, the timing of ischemia/reperfusion period in the present study was carefully chosen and based on the previously published studies (Ballabeni et al., 2002; Naito et al., 2006).

HMG-CoA reductase inhibitors (statins) came into view as the most commonly used agent to lower serum cholesterol levels. On the other hand, recent studies indicate that beneficial actions of statins may not be limited to their cholesterol reducing effect (Takemoto and Liao, 2001). Specifically, acute administration of statins attenuate the damaging effects of transient cerebral (Hayashi et al., 2005; Laufs et al., 2002), myocardial (Di Napoli et al., 2005), and intestinal (Naito et al., 2006) ischemia. These studies indicate that NOS activation induced by statins plays a significant role (Laufs et al., 2002; Shiga et al., 2005). Through improving both eNOS activity and endothelial function, atorvastatin may exert an important effect in order to maintain intestinal contractile response. It has been clearly demonstrated that functional insufficiency of endothelium derived NO occurs after reperfusion of ischemic tissues. That ischemia/reperfusion makes a tremendous impact on endothelium, causing endothelium dysfunction, is well known and ascertained by reduction in the release of NO (Anaya-Prada et al., 2002). Considering the development of endothelial dysfunction, original attribution was to reactive oxygen species mobilized from both reperfused endothelium and activated adherent neutrophils (Cuzzocrea et al., 1998). It is reported that

decreased generation of NO may cause an increase in $O_2^{-\cdot}$ levels and neutrophil infiltration that may in turn augment disruption of intestinal function in ischemia/reperfusion injury (Khanna et al., 2000). Moreover, recent studies suggest that the maintenance of eNOS expression at physiological levels by statins occurs along with a significant inhibition of inflammation (Naito et al., 2006; Naidu et al., 2003). It is well established that statins inhibit Rho/Rho kinase pathway in the vascular smooth muscle. Inhibition of this pathway elevates production and activation of eNOS, counterbalancing the decreased level of NO, whereby contributing to the improvement of endothelial function (Fukata et al., 2001; Noma et al., 2006). Thus, blocking Rho/Rho kinase pathway may cause vasodilation, providing support to the attenuation of contractile dysfunction by atorvastatin that we have observed. The regulation of eNOS by Rho GTPases, therefore, may be an important process among mechanisms underlying the protective effects of atorvastatin we observed in intestinal ischemia/reperfusion.

The increase in MDA content of the terminal ileum following ischemia/reperfusion was significantly inhibited by pretreatment with atorvastatin. Naito et al. (2006) recently report that rosuvastatin, a new HMG-CoA reductase inhibitor, possesses protective effects against oxidative injury and inflammation which are caused by reperfusion. In the present study, we observed not only decreased lipid peroxidation but also concomitant improvement of contractile response in the case of atorvastatin pretreatment. These findings support the notion that atorvastatin ameliorates contractile response due in part to reducing lipid peroxidation of intestinal tissue. Statins have antioxidative effects in *in vivo* and *in vitro* studies. For instance, copper or leukocyte-induced oxidation of low density lipoproteins is blocked by statins (Hayashi et al., 2005). Moreover, beneficial effects of statins in cerebrovascular disorders may be associated with their antioxidative property. Kumagain et al. report that statin treatment prevents SOD immunoreactivity from ischemia-induced decrease in brain. Our observation of reduced lipid peroxidation levels following statin pretreatment may be explained by published discovery which shows that statins inhibit angiotensin II- and NADPH-oxidase induced reactive oxygen species production (Endres and Laufs, 2004). Another explanation for decreased lipid peroxidation is that the effect of atorvastatin may result from its antiinflammatory action, rather than its direct antioxidant activity.

Following pretreatment of animals with atorvastatin prior to inducing intestinal ischemia/reperfusion, reduced lipid peroxidation was coincided with the change in MPO activity, which is known as the index for infiltration of polymorphonuclear neutrophils. Being a potential source of reactive oxygen species, polymorphonuclear neutrophils play a significant role in the development of oxidative tissue injury. Based on substantial amount of studies, MPO activity is elevated in many inflammatory processes, such as ischemia/reperfusion injury. Naito et al. (2006, 2003) reports that MPO activity decreases with statin treatment in reperfused tissue. In agreement with that, in the present study, we observed increased MPO activity in ischemia/reperfusion control group. Furthermore, pretreatment with atorvastatin alleviated ischemia/reperfusion induced increase in MPO activity. These findings implicate that atorvastatin administration can interfere with the interaction between leukocytes and endothelial cells. Furthermore, statins induce immunosuppression that can also be related to the inhibition of interaction between leukocyte function antigen-1 and intracellular adhesion molecule-1 (Arnaud et al., 2005). Statins also reduce activation of the pro-inflammatory transcription factor NF-kB (Martin-Ventura et al., 2005; Haloui et al., 2003), iNOS (Wagner et al., 2002) and COX-2 (Hernandez-Presa et al., 2002), and Rho inactivation (Narumiya et al., 2004). Data obtained by the present study suggest that reactive oxygen species and polymorphonuclear neutrophils are, at least, responsible for reduced contractile response in small intestine subjected to ischemia/reperfusion, while therapy with atorvastatin beforehand minimizes oxidative damage and neutrophil accumulation in ileal muscular cells.

GSH, a principal constituent of the cellular protective mechanisms against free radical-induced tissue injury, is commonly used as index of oxidative stress induced by reactive oxygen species in the process of ischemia/reperfusion. In addition, the level of GSH has been shown to be inversely related to both generation of reactive oxygen species and degree of lipid peroxidation in other inflammatory conditions (Sola et al., 2000). Since it is recyclable and plentiful in majority of cells, GSH is considered as one of the most crucial nonenzymatic antioxidants. Moreover, GSH also recovers other free radical scavengers and antioxidants, such as o-tocopherol and ascorbic acid, from their reduced state. Therefore, measurement of the GSH level can reflect the antioxidative capacity of reperfused tissue (Gunel et al., 1998). Considerable number of studies indicates that GSH contents of various tissues including intestine decrease significantly following ischemia/reperfusion insult (Ozacmak et al., 2005). The improvement of GSH level in animals pretreated with atorvastatin may play a crucial role in prevention of propagation of tissue damage caused by ischemia/reperfusion. Atorvastatin pretreatment seems to afford protection against oxidative stress as observed by the increased levels of GSH in the treated group.

In conclusion, our results suggest that atorvastatin therapy inhibits the infiltration of polymorphonuclear neutrophils and oxidative stress observed in reperfusion injury of small intestine and consequently protects contractile response of ileum. It may be implicated that mechanisms of these actions may be engaged in previously demonstrated beneficial effects of statins on endothelial function by upregulation of eNOS and inhibition of Rho kinase (Takemoto and Liao, 2001). Additionally, atorvastatin-induced reduction of reactive oxygen species could decrease free radical load of vascular system which may provide improvement of endothelium-dependent vasorelaxation; thus, lowering adhesion of macrophages to the vessel wall (Sugiyama et al., 2005). These implications would be among cholesterol-independent effect of statins providing part of the protective effects of these drugs. Overall, we suggest that the therapeutic efficacy of atorvastatin deserves to

be examined for potential clinical application in treatment of conditions related to intestinal ischemia/reperfusion.

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