Acute and Chronic Pretreatment With Atenolol Attenuates Intestinal Ischemia and Reperfusion Injury in Hypercholesterolemic Rats

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Objective: **To evaluate the protective effects of preinjury atenolol (acute v chronic) on apoptosis, contractility, oxidative stress, and inflammatory markers in hypercholesterolemic rats undergoing intestinal ischemia-reperfusion (I/R) injury.**

Design: **Prospective, experimental animal study.**

Setting: **University laboratory.**

Participants: **Male Wistar rats (n = 32).**

Interventions: **Rats were divided into the following 4 groups: 1 group was fed a normal diet (ND) (group ND+NoAT [no atenolol]), and the other 3 groups were fed a high-cholesterol diet (HCD)—group HCD+NoAT, group HCD+ChAT (chronic atenolol, 3 mg/kg/day for 8 weeks), and group HCD+AcAT (acute atenolol, 1.5 mg/kg, given 5 minutes before intestinal clamping). All rats underwent I/R injury. The superior mesenteric artery was clamped for 60 minutes, then opened for 120 minutes (reperfusion). Apoptotic cells and stimulated contractions of ileal segments were examined. Tissue markers of intestinal I/R injury were examined. Intestinal malondialdehyde, superoxide dismutase, and nitrate/nitrite levels were measured.**

Measurements and Main Results: **The chronic atenolol group had fewer apoptotic cells and higher superoxide dismutase activity compared with the other groups. Intestinal contraction was higher in both atenolol pretreatment groups compared with the NoAT groups. Chronic and acute atenolol resulted in lower ileal levels of malondialdehyde and immunolabeling-positive cells (intestinal inducible nitric oxide synthase, endothelial nitric oxide synthase, interleukin-1, and interleukin-8) after I/R injury compared with the no atenolol groups.**

Conclusions: **Both chronic and acute pre-I/R injury treatment with atenolol attenuated I/R injury in this hypercholesterolemic rat model. These findings should encourage future studies of atenolol in hypercholesterolemic patients undergoing procedures with a high risk of intestinal ischemia.**

KEY WORDS: atenolol, ischemia-reperfusion injury, ileum protection, hypercholesterolemia

MESENTERIC ISCHEMIA-REPERFUSION (I/R) injury may occur during abdominal aneurysm repair and cardiopulmonary bypass; both procedures interrupt blood flow to the gut. The intestine particularly is susceptible to I/R injury. The most frequent clinical complications are decreased gastrointestinal motility (ileus) and bacterial translocation, which may lead to the development of secondary infections and multiple organ failure.[1,2]

Hypercholesterolemia is a major risk factor for atherosclerosis, hypertension, and myocardial ischemia. Most patients undergoing cardiovascular surgery are hypercholesterolemic. Hypercholesterolemia is characterized by endothelial nitric oxide synthase (eNOS) uncoupling with formation of superoxide instead of nitric oxide (NO), thus NO production is inhibited.[3] Through heightened oxidative stress or by inactivation of the reperfusion injury salvage kinase pathway, hypercholesterolemia may aggravate apoptotic death and thus contribute to increased organ vulnerability to I/R injury.[3,4] Although the role of oxidative stress and inflammation in I/R injury has been well described,[5-8] its effects in hypercholesterolemia and its modulation by ß-blockers (acute *v* chronic administration) in I/R models has not been addressed. For these reasons, the authors wanted to study the effects of atenolol (AT) in a hypercholesterolemic rat model to more closely mimic the authors' patient population. Such animals may mimic patients with cardiovascular disease who may experience intestinal ischemia during aneurysm repair or bypass.

Therapeutic strategies for I/R injury have focused both on preventing the effects of reactive oxygen species and on downregulating the signal transduction cascades related to the expression of proinflammatory genes.[9-12] AT, a selective β_1 symphathetic receptor blocker, has cytoprotective effects when administered intravenously and improves diminished motility of enteric motor and neural dysfunction caused by intestinal I/R injury in normocholesterolemic rats and rabbits.[13,14] AT also has been found to attenuate the development of pulmonary damage after I/R injury in rats[15] and protein oxidation and lipoxidation damage of cellular proteins in mice.[16]

NO, the free radical produced during I/R, acts as a modulator/messenger with beneficial vasodilatory effects, but excess production of NO (produced by inducible nitric oxide synthase [iNOS]) paradoxically is cytotoxic.[17] NO levels are very difficult to measure directly; therefore, nitrites (NO_2^-) and nitrates (NO_3^-) are measured as a surrogate of NO activity.[6,17]

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In this study, the authors used an intestinal I/R model in hypercholesterolemic rats to examine whether acute or chronic pretreatment with AT would protect against intestinal injury induced by I/R, as measured by markers of cell apoptosis, contractility, oxidative stress, and inflammation. The methodology and animal model that were used to assess the effects of AT on intestinal I/R injury and its effect on other organ systems for intestinal ischemia have been used in previous studies.[15] This same model also was used to assess the protective effect of AT on I/R injury in rats, especially evaluating the preservation of intestinal contractility.[13] The study presented here was the first to examine the effects of I/R injury in hypercholesterolemic animals receiving AT on a chronic basis.

METHODS

This study was performed with approval from the authors' institutional animal ethics committee (2013/77637435-56). All experimental animals received humane care and treatment in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources.[18]

Animals, Experimental Protocols, and Preparation of Terminal Ileum Samples

Male Wistar rats (n = 32), housed at 22°C with a 12-hour light/dark cycle, were divided into the following 4 groups (n = 8 in each): group ND+NoAT (normal diet [ND] with no AT), group HCD+NoAT (high-cholesterol diet [HCD] with no AT), group HCD+ChAT (HCD with chronic use of AT), and group HCD+AcAT (HCD with acute administration of AT). Group ND+NoAT rats were fed a normal standard chow diet (ND) for 20 weeks before the experiment. The other 3 study groups were fed an HCD (98% basal diet with 2% cholesterol) for 20 weeks before the experiment. This duration of an HCD has been found to produce vascular changes that mimic atherosclerotic changes in humans.[19]

The HCD+ChAT group received 3 mg/kg per day of AT via gastric tube for 8 weeks before the experiment. The HCD+AcAT group received a single bolus dose of 1.5 mg/kg of atenolol in the femoral vein 5 minutes before ischemia was induced, as described later in the article. Animals were fasted for 8 hours before the surgery. All groups underwent I/R, as described later in the article.

Rats (375-450 g) were anesthetized with intraperitoneal ketamine (75 mg/kg) and xylazine (10 mg/kg), and then laparotomy was performed. The superior mesenteric artery was clamped off with a metallic clip for 60 minutes (ischemia), then the clamp was released (reperfusion for 120 minutes). One-hour intestinal ischemia was used in this model because this duration previously was found to cause reversible alterations in microvascular permeability, edema, and mucosal injury.[13] The animals were sacrificed 180 minutes after initial clamping and just after collecting the final blood samples for tissue samples to be taken for laboratory analyses (detailed later in the article).

After sacrifice, the ileum was removed immediately and transferred into a petri dish containing Krebs-Henseleit solution of the following composition (in mmol/L): sodium chloride = 118.3, potassium chloride = 4.7, magnesium sulfate = 1.2, potassium phosphate monobasic = 1.22, calcium chloride = 2.5, sodium bicarbonate = 25.0, and glucose = 11.1 that was bubbled with 95% oxygen and 5% carbon dioxide (pH = 7.4, 37°C). Then, the terminal ileum (10-15 cm) was removed, cleaned, and cut into 5 pieces: 2 pieces were used to measure longitudinal muscle contractility, 2 were used for staining for immunohistochemical analysis, and 1 was used for biochemical analysis, as described later in the article.

Intestinal tissue was fixed in a 10% formalin solution for 24 hours, processed, embedded in paraffin, and cut into 5-mm thick sections using a microtome. Sections were prepared for terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end-labeling (TUNEL) assay and immunohistochemistry.

Detection of Apoptotic Cell Death in Situ, Using the TUNEL Method

The authors used standard TUNEL methods to assess for the presence of apoptotic cells (cells undergoing deoxyribonucleic acid fragmentation) after I/R injury to the intestine. These were performed with a commercial immunohistochemical in situ apoptosis detection kit (DeadEnd Colorimetric TUNEL System, Promega Corp., Madison, WI). The cells were treated for 15 minutes with 4% paraformaldehyde, incubated for 10 minutes with 20 μg/mL of proteinase K, and then washed with buffer solution 3 times for 5 minutes each. After treatment with 4% paraformaldehyde for 5 minutes and a second wash for 5 minutes with buffer solution, cells were incubated in terminal deoxynucleotidyl transferase for 1 hour at 37°C. The cells were treated for 10 minutes with a solution that contained 22% sodium chloride and 11% sodium citrate and then were treated for 5 minutes with 3% hydrogen peroxide (Lab Vision UltraVision LP Detection System TA-015-HP; ThermoFisher Scientific, Waltham, MA) to inhibit endogenous peroxidase. The cells were washed with buffer solution at room temperature for 10 minutes and then were incubated with antistreptavidinperoxidase enzyme for 30 minutes. The cells then were washed with buffer solution, stained with 3,3'-diaminobenzidine, and observed using a microscope for the TUNEL reaction.[20] Staining was examined independently by 2 histologists (I.T. and I.A.), who had no information about the origin of the samples. TUNEL-positive cells were counted in each visual field and reported as a percentage of total cells.

Ileum Longitudinal Muscle Contractility

The pieces of ileum used to measure longitudinal muscle contractility were suspended in a standard organ chamber that was perfused continuously with 20 mL of oxygenated Krebs solution. At the beginning of each experiment, potassium chloride (30 mM) was added to the organ chamber and the contractions were recorded as a reference (maximum) response. Then, contractions were measured in response to various concentrations (10^{-9} to 10^{-4} M) of the depolarizing agent acetylcholine hydrochloride. Starting with the most dilute acetylcholine solution (10^{-9}), the acetylcholine solutions were pipetted into the organ bath in a cumulative fashion at equal intervals, and contractions were measured with a digital