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Cardioprotective effects of luteolin during ischemia/reperfusion injury in rats

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ABSTRACT

Background: Antioxidants effectively reduce ischemia and reperfusion injury. We examined the cardioprotective effects of luteolin, a flavonoid that exhibits antioxidant properties and is widely available in many fruits and vegetables, in rats subjected to myocardial ischemia and reperfusion injury.

Methods and Results: Rats were subjected to myocardial ischemia or reperfusion injury, to evaluate the antiarrhythmic effects of luteolin. Myocardial infarct size was determined histochemically with triphenyltetrazolium chloride staining of the left ventricle. Luteolin was administered intravenously 15 min before occlusion of coronary artery. The incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF) and mortality during myocardial ischemia were significantly reduced by luteolin (10 μg/kg). Similarly, luteolin (1 μg/kg) reduced ventricular arrhythmias and mortality during the reperfusion phase. At the same time, pretreatment with luteolin decreased plasma lactate dehydrogenase (LDH) and nitric oxide (NO) levels. Luteolin (10 μg/kg) significantly reduced cardiac infarct size, as well as malondialdehyde (MDA) production in myocardial ischemia and reperfusion injury tissue samples. Luteolin also downregulated inducible nitric oxide synthase (iNOS) protein and mRNA expression, but did not significantly alter neuronal nitric oxide synthase (nNOS) or endothelial nitric oxide synthase (eNOS) expression.

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Conclusions: Luteolin is capable of protecting the myocardium against ischemia and reperfusion injury. The actions of luteolin are at least partly mediated through the downregulation of NO production and its antioxidant properties.

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Key Words: Cardioprotective agent; Luteolin; Ischemia; Reperfusion; Arrhythmia; Infarction; Nitric oxide; Antioxidant

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Introduction

Ischemia is characterized in part by low tissue oxygen tension. It is well documented that salvage of the ischemic myocardium is dependent upon timely reperfusion;[1] it is likely that the very events critical for survival may, in fact, lead to further tissue injury.[2,3] Various evidence from investigations into the myocardium suggests that reactive oxygen species, including superoxide radicals, hydrogen peroxide, hydroxyl radicals and singlet oxygen contribute to the pathophysiology of myocardial ischemia and reperfusion injury.[3,4] These reactive oxygen species, which are formed within the myocardial ischemia and first few moments of reperfusion, are known to be cytotoxic to surrounding cells.[5] Thus, myocardial ischemia and reperfusion injury induce ventricular arrhythmias, resulting in circulation collapse and sudden death.[6-8] Effective inhibition of reactive oxygen species production or elimination of oxygen-derived free radicals is therefore an important strategy for the treatment of ventricular arrhythmia and myocardial infarction caused by myocardial ischemia or reperfusion injury.[9,10]

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Luteolin is one of the most widely distributed flavonoids, a group of naturally occurring polyphenolic compounds found in many fruits and vegetables.[11,12] It has been reported in the literature that luteolin has a wide range of biological and pharmacological properties including antineoplastic,[13-15] antihepatotoxic, antiallergic,

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antiosteoporotic,[16] antidiabetic,[17] and anti-inflammatory activity,[18] antiplatelet and vasodilatory activity,[12] as well as antioxidant effects.[19] At low concentrations (IC_{50} value of 0.96 μM), luteolin has also been shown to inhibit xanthine oxidase activity, which has been implicated in tissue-related oxidative injury after ischemia-reperfusion.[20] Recently, in human melanoma HMB-2 cells, luteolin has shown a concentration-dependent inhibitory activity toward DNA damage induced by H_2O_2.[21] In addition, luteolin has been shown to significantly enhance left ventricular pressure and the global and relative coronary flow in Langendorff rabbit hearts subjected to repetitive myocardial ischemia.[22] The antioxidant properties of luteolin prompted us to investigate whether luteolin is capable of exerting beneficial effects during myocardial ischemia and reperfusion injury. Our present study therefore evaluated the cardioprotective effects of luteolin during myocardial ischemia and reperfusion injury in anesthetized rats subjected to transient coronary artery occlusion and reperfusion. Animals were pretreated with or without luteolin before coronary artery ligation. The severity of myocardial ischemia and reperfusion-induced arrhythmias, including the incidence and duration of ventricular tachycardia (VT) and ventricular fibrillation (VF), mortality and infarct size, were compared between the groups of animals.

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Methods

Animals

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We used male Sprague-Dawley rats (National Lab. Animal Breeding and Research Center, Taipei, Taiwan) weighing 250~300 g. The animals were housed in a room under controlled temperature (24±1°C) and humidity (55±5%) conditions and subjected to a 12:12 h light-dark cycle. They were allowed free access to food and water.

Surgical procedure

Myocardial ischemia and reperfusion injury were induced by a temporary occlusion of the left main coronary artery in procedures as described previously.[23] Briefly, male Sprague-Dawley rats were anesthetized with intraperitoneal urethane (1.25 g/kg) and placed on an operating table. The trachea was cannulated for artificial respiration and the jugular vein was cannulated for drug administration. Polyethylene catheters (PE-50) were inserted into the common carotid artery for continuous monitoring of heart rate and arterial blood pressure by a Statham P23 XL transducer and displayed on a Gould RS-3400 physiological recorder (Gould, Cleveland, OH, USA). A standard lead-1 electrocardiogram (ECG) was recorded via silver electrodes attached

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to the extremities.

After tracheotomy, the animals were ventilated with room air by a respirator for small rodents (Model 131, NEMI, U.S.A.) using a stroke volume of 15 mL/kg body weight and at a rate of 60 strokes/min to maintain normal P_{O_2}, Pc_{O_2} and pH parameters (blood gas analyzer, GEM-5300 I.L. CO, USA). The chest was opened by a left thoracotomy, followed by sectioning of the fourth and fifth ribs, approximately 2 mm to the left of the sternum. The heart was quickly expressed out of the thoracic cavity, inverted and a 6/0 silk ligature was placed around the left main coronary artery. The heart was repositioned in the chest and the animal was allowed to recover for 15 min. Animals in which the procedure produced arrhythmia or a sustained decrease in BP to less than 70 mmHg were not included in the study.

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A small plastic snare formed from a Portex P-270 cannula was threaded through the ligature and placed in contact with the heart. The coronary artery was then occluded by tightening the ligature and reperfusion was achieved by releasing the tension applied to the ligature (operated groups). Successful ligation of the coronary artery was validated by observation of a decrease in arterial pressure and ECG changes (increase in R wave and ST segment elevation) indicative of ischemia. Sham-operated animals underwent all surgical procedures, except that the silk passing around the left coronary artery was not tied.[24]

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Evaluation of arrhythmia

For evaluating the effects of luteolin during myocardial ischemia or reperfusion injury, the coronary artery was occluded for 30 min or 5 min followed by 30 min reperfusion. In previous studies, the majority of myocardial ischemic arrhythmias occurred during the first 30 min of ligation,[25] while a 5-min period of ischemia followed by a 30-min reperfusion period is associated with the highest incidence of reperfusion-induced arrhythmias.[26] Before and during the ischemia or reperfusion period, heart rate, blood pressure, and ECG changes were recorded simultaneously on a personal computer with waveform analysis software (AcqKnowledge, Biopac System, Goleta, California, USA). Ventricular ectopic activity was evaluated according to the diagnostic criteria advocated by the Lambeth Convention.[27] The incidence and duration of ventricular tachyarrhythmias, including VT and VF, were determined in surviving and nonsurviving animals. In rats with irreversible VF, the duration of VF was recorded up until when BP fell to <15 mmHg.

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Estimation of myocardial injury

Myocardial cellular damage was evaluated by measuring lactate dehydrogenase (LDH) activity in plasma. LDH released from necrotic tissue was determined from arterial blood plasma drawn from the carotid catheter at the end of ischemia and reperfusion injury and collected in polyethylene tubes containing 50 μl heparin (250 IU). LDH

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activity was measured according to the method of Tsai et al.,[28] spectrophotometrically following the rate of conversion of NADH to NAD^+ at 340 nm with a commercially available assay kit (Sigma, St Louis, MO).

Plasma NO metabolite levels (NOx^-)

Arterial blood samples were drawn from the carotid catheter at the end of ischemia or reperfusion phase. The assay has been previously described in detail.[29] NO production was estimated from the amounts of nitrite ($NO2^-$) and nitrate ($NO3^-$) in deproteinized plasma samples assayed with a commercially total nitric oxide assay kit (Stressgen, Ann Arbor, MI). $NO3^-$ was calculated by first reducing $NO3^-$ into $NO2^-$ in the presence of Cd, and $NO2^-$ was determined by a colorimetric assay based on the Griess reaction. The measurement of NOx^- levels has been found to be a reliable technique to determine the synthesizing capacity of NOS in the heart.

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Estimation of myocardial infarct size

Only those rats that survived one hour of coronary ischemia and three hours of reperfusion were included for evaluation of the infarct zone. Occluded zone and infarct zone sizes in rat heart were determined following the procedures previously described by Hung et al. Prolonged ischemia and reperfusion durations were required to produce an infarct area for pathological evaluation.[30] At the end of the experiment, the coronary artery was re-occluded and injected intravenously with 2.0 mL 3%

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methyl blue to denote the area at risk. With this technique, the previously non-ischemic area appears blue whereas the area at risk remains unstained. The latter region was cut out, weighed and the occluded zone was expressed as the percentage of the total ventricular weight. Thereafter, ventricular tissue was sliced into 1 mm sections and incubated in tetrazolium dye (2,3,5-triphenyltetrazolium chloride [TTC, 1%; Sigma, USA] in normal saline) at 37°C for 40 min in darkness. Sections were then placed in a solution of 10% formaldehyde in saline for 2 days before excising infarct (white) tissue. The weight of infarct tissue was expressed as a percentage of the total ventricle or the area at risk.

Western blot analysis

Rats were perfused with saline and the hearts were prepared for Western blot analysis. Heart tissue was homogenized in Laemmli lysis buffer containing protease inhibitors (10 μL / 0.2 g tissue weight, SIGMA, St. Louis, MO). Protein concentrations in each sample solution were determined using a protein assay kit (BCA kit; Pierce, Rockford, IL) and the samples were stored at –80°C until use. Aliquots containing 120 μg of protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (IPVH00010; Millipore Corp., Bedford, MA). Western blot analysis of NOS protein was performed as previously described.[30] Protein bands were transferred onto a

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polyvinylidene difluoride membrane (IPVH00010; Millipore Corp., Bedford, MA) and probed for inducible nitric oxide synthase (iNOS) (1:1000 [catalog no. N32020; Transduction Laboratories, Lexington, KY]), neuronal nitric oxide synthase (nNOS) (1:1000 [catalog no. N41520; Transduction Laboratories, Lexington, KY]), endothelial nitric oxide synthase (eNOS) (1:1000 [catalog no. N30020; Transduction Laboratories, Lexington, KY]) and 1:2000 actin (sc-1616 Santa Cruz Biotechnology, Santa Cruz, CA) by incubation in the primary antibody, followed by a horseradish peroxidase–conjugated secondary antibody 1:1000 (catalog no M15345 fon NOS; Transduction Laboratories, Lexington, KY and catalog no 7074 for actin; Cell Signaling Technology, Inc., USA). Blots were visualized using the western lightning chemiluminescence reagent (PerkinElmer Life Science, Inc., Boston) according to the manufacturer's directions, and were exposed to x-ray film.

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Reverse transcription polymerase chain reaction (RT-PCR)

nNOS, eNOS and iNOS mRNA were detected in the occluded zone of the heart by RT-PCR, as previously described.[30] Total RNA was extracted from the heart tissue with RNase Maxi kits (Qiagen, Valencia, CA,USA). First-strand cDNA synthesis was then performed with the use of 5 μg of total RNA, oligo (dT) primer (BRL), and Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RT-PCR was carried out in O' in 1 DNA polymerase

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solution at 50°C for 60 min, followed by enzyme inactivation at 72°C for 15 min. The primer sequences were as follows:

nNOS forward primer: 5'-TTCCGAAGCTTCTGGCAACAGCGACAATTT-3',

nNOS reverse primer: 5'-AGATCTAAGGCGGTTGGTCACTTC-3',

iNOS forward primer: 5'-TCACGACACCCTTCACCACAA-3',

iNOS reverse primer: 5'-CCATCCTCCTGCCCACTTCCTC-3',

eNOS forward primer: 5'-TGGGCAGCATCACCTACGA-3',

eNOS reverse primer: 5'-TCCCGAGCATCAAATACCT-3',

β-actin forward primer: 5'-CCAGAGCAAGAGAGGCATCCTG-3',

β-actin reverse primer: 5'-GCCGATAGTGATGACCTGACCGT-3'.

The amplification procedure consisted of initial denaturation at 95°C for 5 min, followed by cycle parameters of denaturation at 95°C for 1 min, annealing at 60°C for 1 min, with controlled extension at 72°C for 1 min, for 35 cycles. The amplified products were separated by gel electrophoresis in 1.5% agarose gel containing 0.5 mg/ml ethidium bromide. Each set of PCRs included control samples run without RNA or in which the RT step was omitted. The RT-PCR procedure was highly reproducible under the present experimental conditions.

Assessment of lipid peroxidation

The MDA content of heart tissue homogenates were measured with a commercially

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available assay kit (Bioxytech MDA 586; Oxis Research, Portland, OR).[31] Colorimetric analysis was performed at 586 nm, MDA production was read from a standard curve and corrected for tissue protein content (nmoL/mg protein). Bradford's method was used to assess protein concentration.[32]

Drug administration

Luteolin was purchased from the Sigma Chemical Company (St. Louis, Mo. USA) and luteolin solution was freshly prepared before administration. Luteolin (0.01, 0.1, 1 or 10 μg/kg) or vehicle (dimethyl sulfoxide-0.9% NaCl, 1:10^4; v/v) was infused via a jugular vein 15 min before coronary artery occlusion. Rats injected with vehicle were used as control. At the given concentration, vehicle had no effects on ischemia- or reperfusion-induced arrhythmia and infarction. Animals were randomly allocated to either drug treatment or vehicle administration.

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Statistics

Data were expressed as mean ± standard error of mean (SEM). Between-group differences in blood pressure, heart rate, duration of VT and VF, infarct size, plasma LDH and NO levels were assessed by analysis of variance (ANOVA) followed by the Newman-Keuls test. The difference in the percentage incidence of VT, VF and mortality was analyzed with a χ^2 test. $P < 0.05$ was considered to be statistically significant.

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Results

Hemodynamic changes during coronary artery occlusion

Jugular vein injection of luteolin did not change mean arterial pressure or heart rate in rats subjected to myocardial ischemia or reperfusion injury. No significant differences were recorded between the vehicle- and luteolin-treated rats (data not shown).

Myocardial ischemia-induced rhythm disturbances

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The effects of luteolin on coronary ligation-elicited arrhythmias in anesthetized rats are shown in Table I. In the vehicle-treated group, severe ventricular arrhythmias occurred at 6–7 min and peaked at 8–12 min, and had normally subsided within approximately 15 min after coronary occlusion. Among the 15 rats in the vehicle-treated group, 13 animals (87%) exhibited VT (42.9 ± 12.8 sec in duration) and 10 animals (67%) exhibited VF (81.0 ± 23.7 sec in duration). However, administration of luteolin at a dose of 10 μg/kg 15 min prior to coronary occlusion significantly reduced the incidence of VT (29%) and VF (13%) as well as the duration of VT (2.7 ± 2.5 sec) and VF (3.7 ± 3.7 sec). The mortality rate was significantly decreased from 53% to 0% in rats treated with 10 μg/kg luteolin.

Myocardial reperfusion-induced rhythm disturbances

The effects of luteolin on myocardial reperfusion-elicited arrhythmias in anesthetized rats are shown in Table II. The severity of reperfusion-induced arrhythmias is

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critically dependent on the duration of the preceding period of ischemia. In this study, we selected a 5-min period of ischemia followed by a 30-min period of reperfusion in order to produce maximal effects of rhythm disturbance.[5] During myocardial reperfusion injury, compared with vehicle-treated rats, the groups of rats administered 0.1 μg/kg and 1 μg/kg of luteolin had significantly lower durations of VT (15.7 ± 5.2 sec vs. 2.0 ± 0.9 sec and 0.6 ± 0.6 sec, respectively; p<0.05) and VF (77.8 ± 18.5 sec vs. 0.7 ± 0.7 sec and 0.0 ± 0.0 sec, respectively; p<0.05), but the incidences of VT (13% vs. 67%, p<0.05) and VF (0% vs. 67%, p<0.05) were significantly reduced only by the 1 μg/kg luteolin dose. The mortality rate was lowered from 53% to 0% by 1 μg/kg luteolin.

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Myocardial injury

Biochemical indication of cellular damage was examined by measuring LDH leakage into plasma at the end of the myocardial ischemia and reperfusion injury phase. The effects of luteolin on the changes in LDH activity in plasma during ischemia and reperfusion injury are shown in Figure 1. Low LDH activity in the plasma was recorded in the sham-operated animals (52.8 ± 13.9 U/L; n=6). However, after myocardial ischemia and reperfusion injury, there was a large increase in LDH levels from baseline in the plasma of rats given vehicle (from 124.5 ± 18.1 to 167.8 ± 20.4 U/L, respectively; n=6). In contrast, the administration of luteolin dose-dependently

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reduced LDH release.

Plasma NO concentration

The effects of luteolin on NO concentrations are shown in Figure 2. NO release was measured by the presence of nitrite (NO_2^-) and nitrate (NO_3^-) in plasma. In sham-operated rats, plasma NO was 4.76 ± 1.10 μmoL/L (n=6). In the operated animals without luteolin treatment, the plasma NO concentration was 7.31 ± 1.00 μmoL/L during myocardial ischemia (n=6) and 7.99 ± 1.03 μmoL/L during reperfusion (n=6). Administration of luteolin decreased NO release in a dose-dependent manner during both the myocardial ischemia and reperfusion phases.

Myocardial infarct size

The effects of luteolin on myocardial infarct size are shown in Table III. There was no significant difference in the size of area at risk between the vehicle- (68.3 ± 2.9%) and luteolin- (69.9 ± 0.9%) treated groups. This indicated that in each group, a similar amount of tissue was at risk by occlusion of the left coronary artery. In the vehicle-treated group, the infarct size was 18.6 ± 1.5% of the area at risk. If 10 μg/kg luteolin was administered prior to occlusion, the infarct size was significantly reduced to 10.0 ± 1.2% of the area at risk.

NOS protein expression

It was noticed that levels of iNOS, eNOS, and nNOS protein were similar between the

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non-occluded and occluded zones of sham-operated rats. As shown in Figure 3, the density of NOS protein expression was normalized with β-actin from the same samples. Cardiac ischemia for 1 hour and reperfusion for 3 hours induced iNOS expression, while administration of luteolin 10 μg/kg prior to the operation significantly suppressed iNOS induction in the occluded zone. In contrast, levels of eNOS and nNOS protein expression were not significantly different between non-occluded and occluded heart tissue after ischemia and reperfusion injury, with or without luteolin treatment. Each value represents the mean of 6 individual experiments.

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NOS mRNA expression

Luteolin at the dose of 10 μg/kg reduced the iNOS mRNA signal in the occluded zone compared with the vehicle-treated group. However, eNOS and nNOS mRNA expression in the non-occluded and occluded zones were not significantly different between the luteolin-treated group and the vehicle-treated group (Fig. 4).

MDA levels

In vehicle-treated rats, after 1 hour of myocardial ischemia and 3 hours of reperfusion, MDA levels in heart tissue were significantly elevated compared to those in sham-operated rats (336.4 ± 12.2 nM/mg vs. 294.2 ± 2.7 nM/mg) ($P < 0.05$). In the luteolin-treated group, MDA levels were significantly attenuated (226.6 ± 18.7

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nM/mg) ($P < 0.05$) compared with vehicle-treated rats (Fig. 5).

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Discussion

In this study, we showed that administration of luteolin at 1 μg/kg or 10 μg/kg significantly suppressed the incidence and duration of VT and VF and completely prevented mortality during myocardial ischemia and reperfusion injury. These results indicate that luteolin exhibits cardioprotective effects against myocardial ischemia and reperfusion injury. This is consistent with the finding that pretreatment with luteolin decreases carotid blood LDH levels, which serve as an indicator of cellular damage, during the same period. In addition, in animals subjected to 1 hour of coronary artery occlusion and 3 hours of reperfusion, the cardiac infarct zone was reduced by pretreatment with luteolin.

Our results also showed that plasma NO concentrations after myocardial ischemia and reperfusion injury were significantly decreased in a dose-dependent manner in the luteolin-treated groups compared with vehicle-treated rats. NO is a small, gaseous biological active messenger with a broad range of physiological and pathological actions.[33] NO possesses vasodilative effects,[34] antiplatelet activity[35] and anti-inflammatory activity,[36] which are all beneficial for cardiac-related improvement after myocardial ischemia and reperfusion injury. However, the reputed cardiotoxic or cardioprotective role of NO during myocardial ischemia and reperfusion injury generates considerable debate. NO can inhibit mitochondria function and break

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single-stranded DNA. In addition, NO and superoxide radicals can rapidly combine to form a strong reactive metabolite, peroxynitrite, which is a potent oxidant that can potentially cause membrane lipid peroxidation and lead to myocardial dysfunction.[37,38] During the myocardial ischemia and reperfusion phase, luteolin suppression of NO production might prevent NO from interacting with superoxide radicals, thereby preventing free radical injury. The family of NOS enzymes comprises the constitutive neuronal NOS (nNOS), eNOS and iNOS enzymes. Beneficial effects of eNOS include vasodilation, inhibition of platelet aggregation and polymorphonuclear neutrophil (PMN) adhesion, whereas nNOS and iNOS appear to be deleterious.[39,40] Recently, Marieke et al. showed that luteolin inhibited NO production and reduced the expression of iNOS in lipopolysaccharide-stimulated NR8383 macrophages.[41] In this study, we found that iNOS protein was induced in rat heart tissue after myocardial ischemia and reperfusion injury. Pretreatment with luteolin suppressed iNOS protein expression in these conditions. However, there was no significant difference in nNOS and eNOS protein expression between the luteolin-treated group and the vehicle-treated group. Further investigations showed a similar result for mRNA expression. Luteolin downregulated iNOS mRNA expression, without influencing nNOS or eNOS mRNA expression. This suggests that the effect of luteolin on iNOS expression was through transcription. However, Northern

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analysis revealed that iNOS expression increases in both non-occluded zones as well as occluded zones, which does not correlate with the induction of protein levels seen only in occluded zones as according to Western analysis. Our results suggest that iNOS mRNA is expression in both non-occluded and occluded zones, but the myocardium expresses iNOS transcription only after ischemia and reperfusion injury.

Investigations support the contention that reactive oxygen species play an important role in the pathophysiology of myocardial ischemia and reperfusion injury.[42,43] The interaction of oxygen-derived free radicals with cell membrane lipids and essential proteins leads to metabolic, electrophysiologic, and functional alterations of the myocardium, which may induce potentially lethal ventricular arrhythmia and myocardial necrosis.[6,44] Luteolin is believed to be an antioxidant or a free radical scavenger in the biological system.[21-23] In support of this contention, we found that levels of MDA, a lipid peroxidation product regarded as a presumptive marker for oxidative stress, were decreased in heart tissue after myocardial ischemia and reperfusion injury in rats administered luteolin. It is suggested that the mass production of oxygen-derived free radicals during myocardial ischemia and reperfusion period may be arrested by the antioxidant activity of luteolin.

In conclusion, our study presents the first *in vivo* evidence that pretreatment with luteolin could effectively protect the myocardium against myocardial ischemia and

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reperfusion-induced cardiac injury. We speculate that the beneficial cardioprotective effects of luteolin are due to the enhancement of antioxidant activity and reduction in NO production.

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Acknowledgment

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Figure legends

Figure 1. Effects of luteolin on plasma LDH activity at the end of the myocardial ischemia (A) and reperfusion (B) phase. Each experimental group included six rats. Results are expressed as mean ± SEM values. Statistical analysis was performed by one-way ANOVA followed by the unpaired Student's *t*-test (*, $p<0.05$ versus vehicle). Note that luteolin dose-dependently decreased plasma LDH activity in both ischemia and reperfusion rats.

Figure 2. Effects of luteolin on plasma NO at the end of the myocardial ischemia (A) and reperfusion (B) period in rats. NO release was measured by the presence of nitrite and nitrate in the plasma. Each experimental group included six rats. Values are expressed as mean ± SEM values. * $p < .05$ compared with the vehicle.

Figure 3. Western blot analyses of heart tissue in rats after myocardial ischemia and reperfusion injury for iNOS, eNOS, nNOS and β-actin. Rats were treated with vehicle or luteolin 10 μg/kg 15 min before coronary artery occlusion. Data were normalized with β-actin and expressed as percentage rates. Each value represents the mean of six individual experiments. Results are expressed as mean ± SEM values. * $p < .05$ compared with the vehicle. S = sham group, non-ischemic operated rats; V = vehicle-treated group; L = luteolin-treated group.

Figure 4. Analysis of iNOS, eNOS, nNOS and β-actin mRNA expression of heart

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tissue in rats after myocardial ischemia and reperfusion injury. Rats were treated with vehicle or luteolin 10 μg/kg 15 min before coronary artery occlusion. S = sham group, non-ischemic operated rats; V = vehicle-treated group; L = luteolin-treated group.

Figure 5. Effects of luteolin on MDA production in rat heart tissue after myocardial ischemia and reperfusion injury. Each value represents the mean of six individual experiments. Values are expressed as mean ± SEM values. * $p < .05$ compared with vehicle.

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Table 1. Effect of luteolin on coronary ligation (30 min) induced arrhythmias in anesthetized rats

		Ventricular Tachycardia		Ventricular Fibrillation		Mortality
	n	Incidence (%)	Duration (s)	Incidence (%)	Duration (s)	(%)
Sham						
Vehicle	4	—	—	—	—	—
Luteolin 1 μg/kg	4	—	—	—	—	—
Operated (Coronary ligation)						
Vehicle	15	87	42.9±12.8	67	81.0±23.7	53
Luteolin 0.01 μg/kg	8	50	23.0±13.5	38	33.6±20.1	25
0.1 μg/kg	8	50	7.7±4.1	25	12.7±12.3	13
1 μg/kg	8	25*	5.4±4.0*	25	3.1±2.4*	0*
10 μg/kg	8	25*	2.7±2.5*	13*	3.7±3.7*	0*

Vehicle is 0.01% DMSO in normal saline; n = number of experiments; values for duration of VT and VF are shown as the mean ± S.E.M.

* Statistical difference at the level of $p < 0.05$ as compared with vehicle.

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Table 2. Effect of luteolin on reperfusion (30 min) induced arrhythmias in anesthetized rats

		n	Ventricular Tachycardia		Ventricular Fibrillation		Mortality
			Incidence (%)	Duration (s)	Incidence (%)	Duration (s)	(%)
Sham							
Vehicle		4	—	—	—	—	—
Luteolin	1 μg/kg	4	—	—	—	—	—
Operated							
Vehicle		15	67	15.7±5.2	67	77.8±18.5	53
Luteolin	0.01 μg/kg	8	50	3.7±2.4	38	38.3±34.2	25
	0.1 μg/kg	8	50	2.0±0.9*	13*	0.7±0.7*	0*
	1 μg/kg	8	13*	0.6±0.6*	0*	0.0±0.0*	0*

Vehicle is 0.01% DMSO in normal saline; n = number of experiments; values for duration of VT and VF are shown as the mean ± S.E.M.

* Statistical difference at the level of $p < 0.05$ as compared with vehicle.

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Table 3. Weights and Size of Area at Risk (n=6)

	Vehicle	Luteolin (10 ug/kg)
LV weight (g)	0.70±0.02	0.70±0.04
Area at risk (g)	0.50±0.03	0.49±0.03
Area at risk/LV (%)	68.3±2.9	69.9±0.9
Inarct size (g)	0.10±0.01	0.05±0.01*
Infarct size/LV (%)	11.8±1.2	7.0±0.9*
Risk zone infarcted (%)	18.6±1.5	10.0±1.2*

Data are mean ± SEM.

LV = left ventricular;

* P<0.05 compared with control group.

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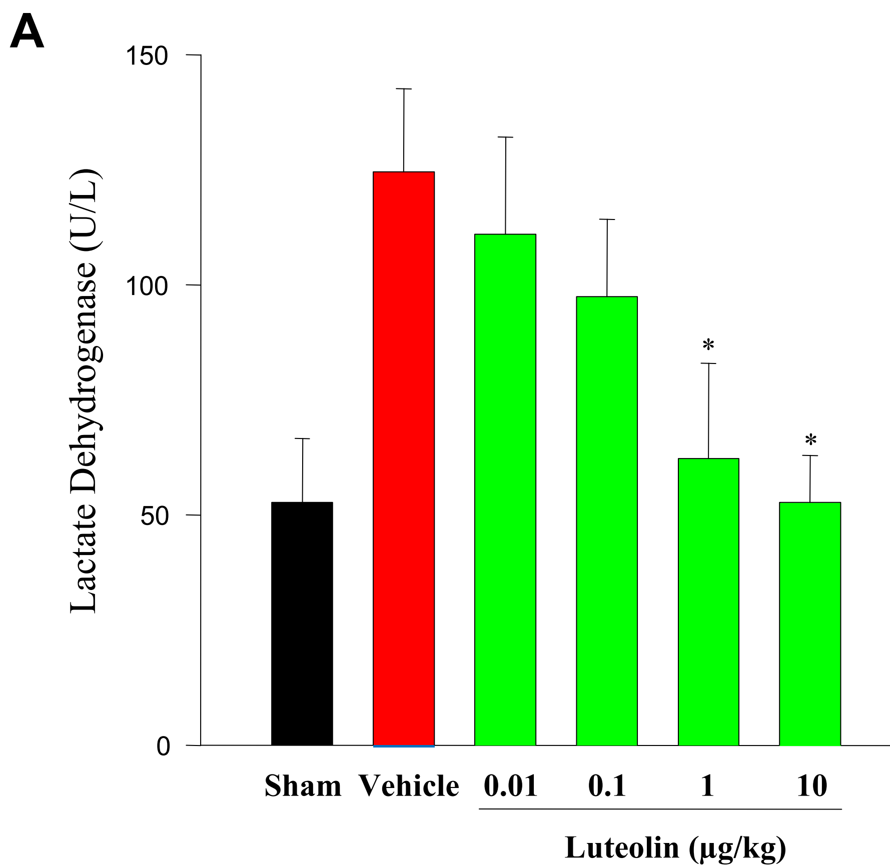

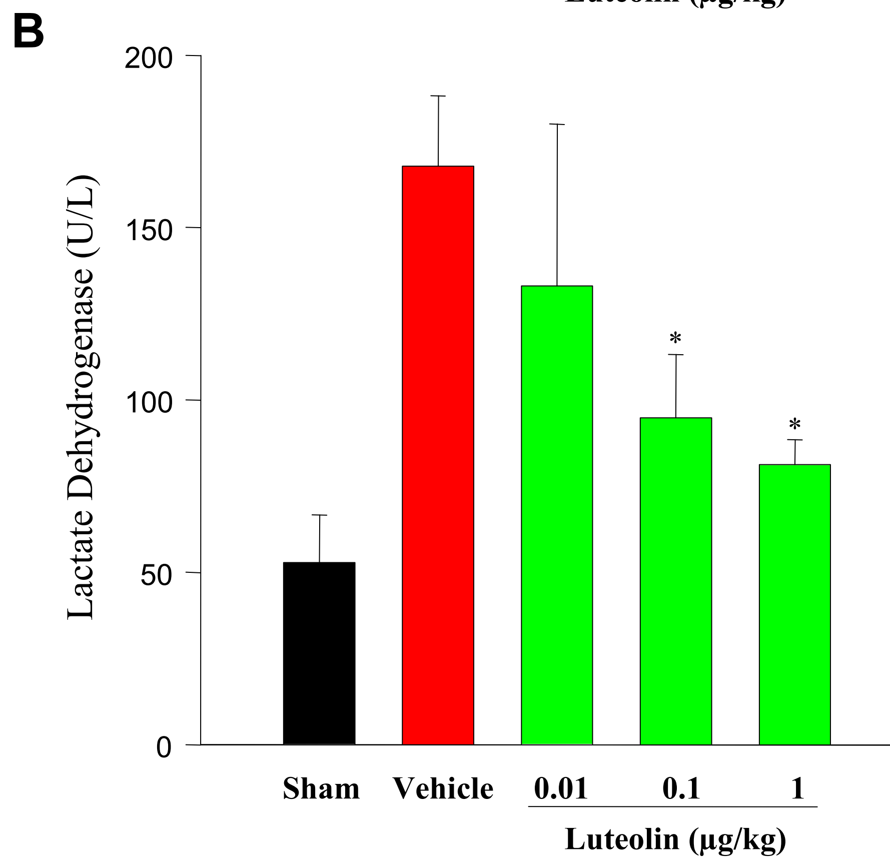

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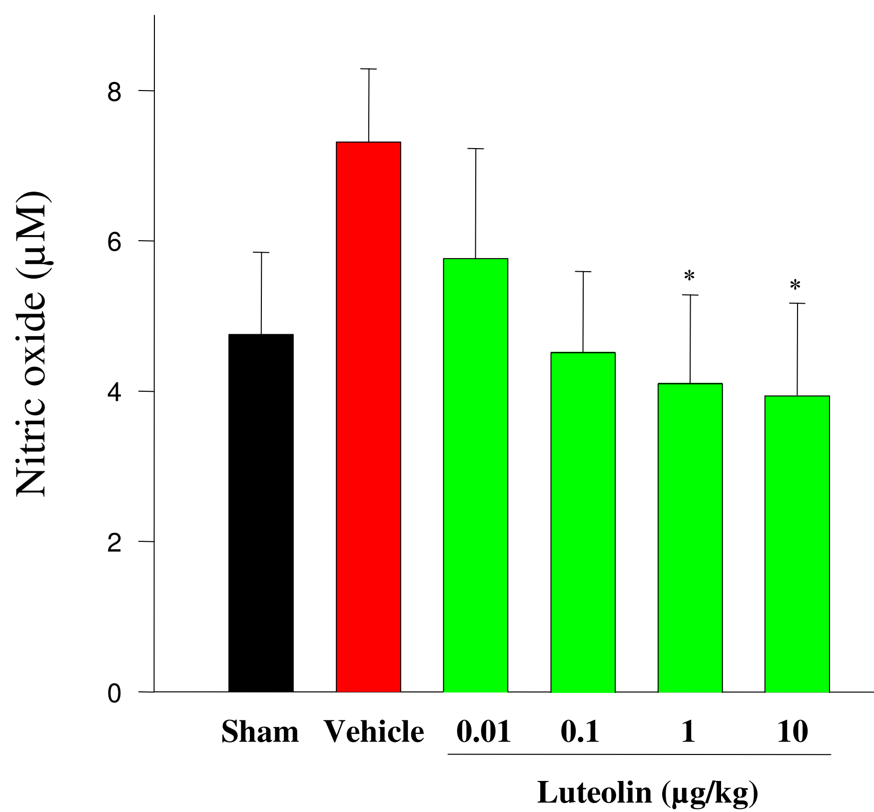

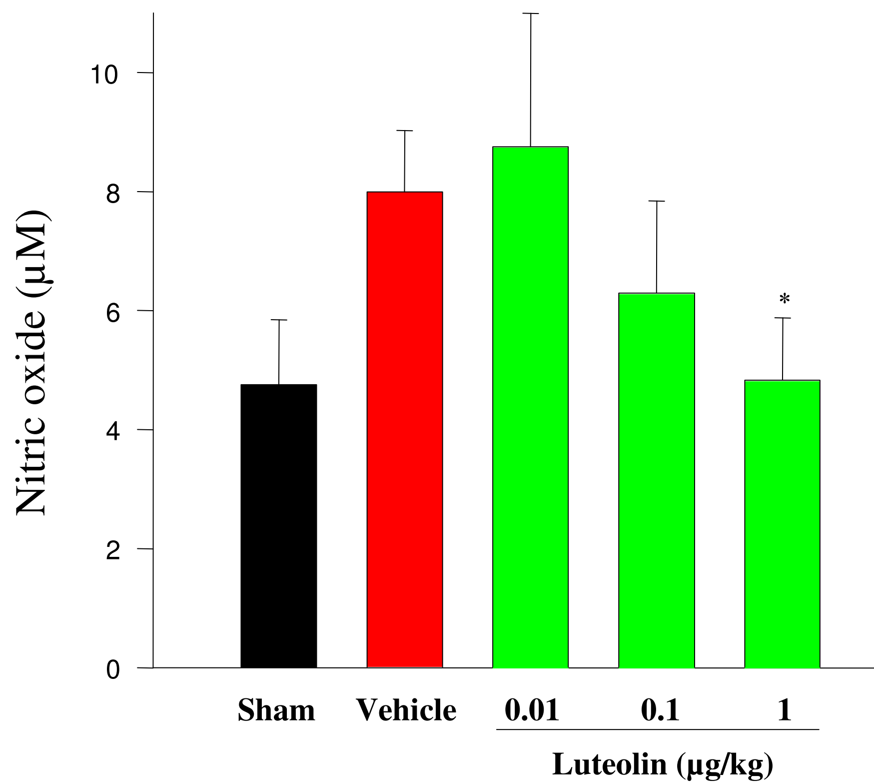

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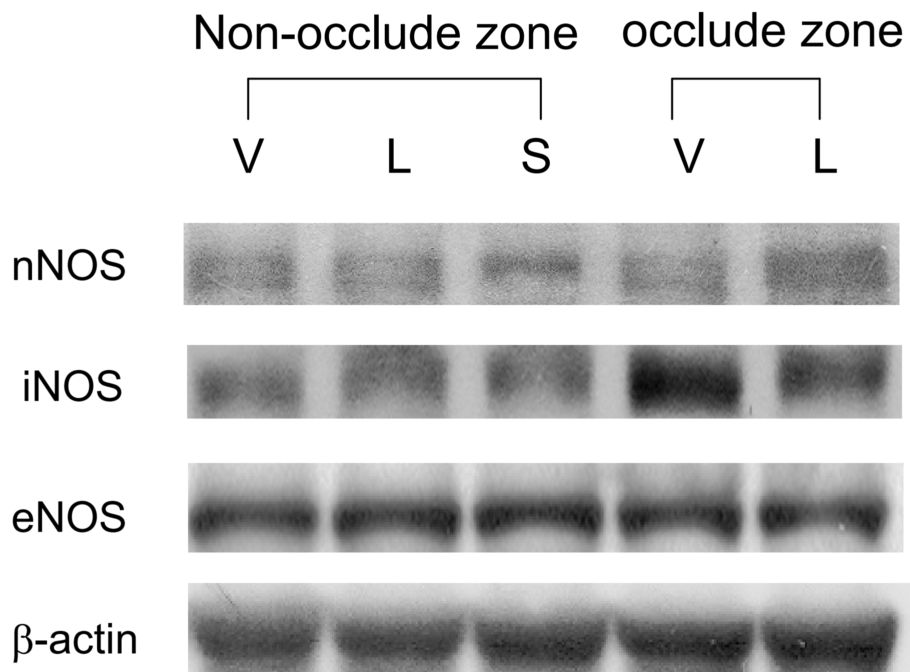

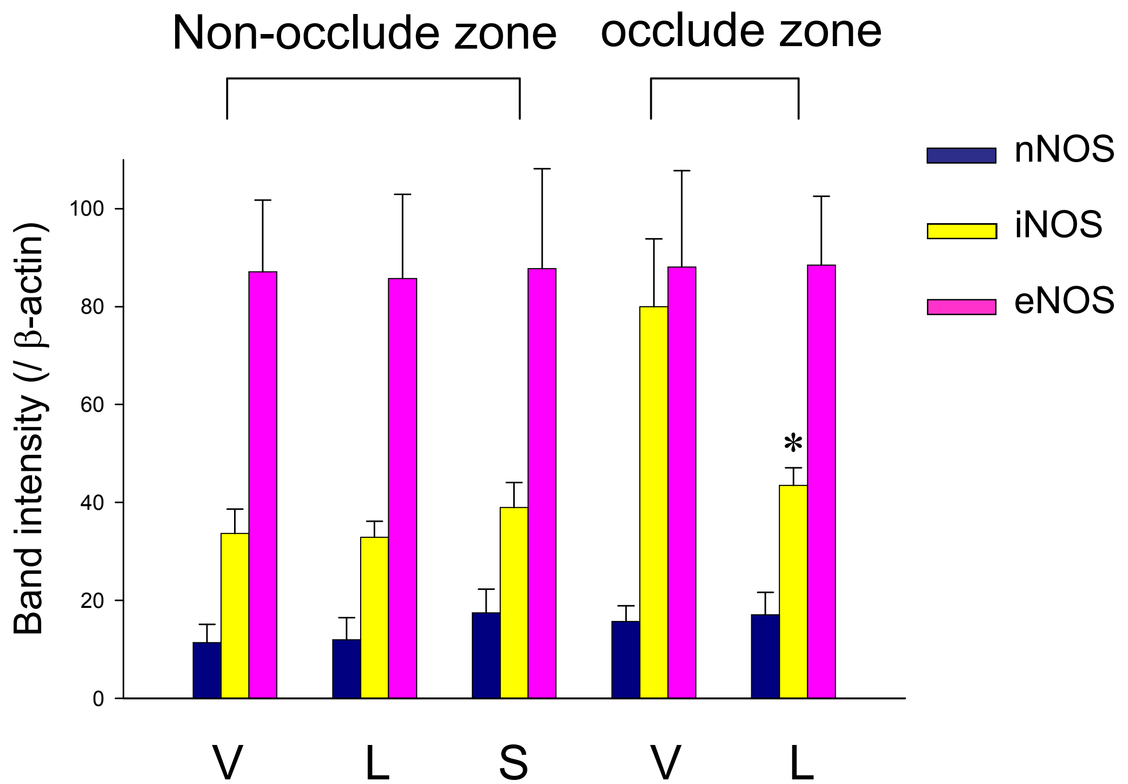

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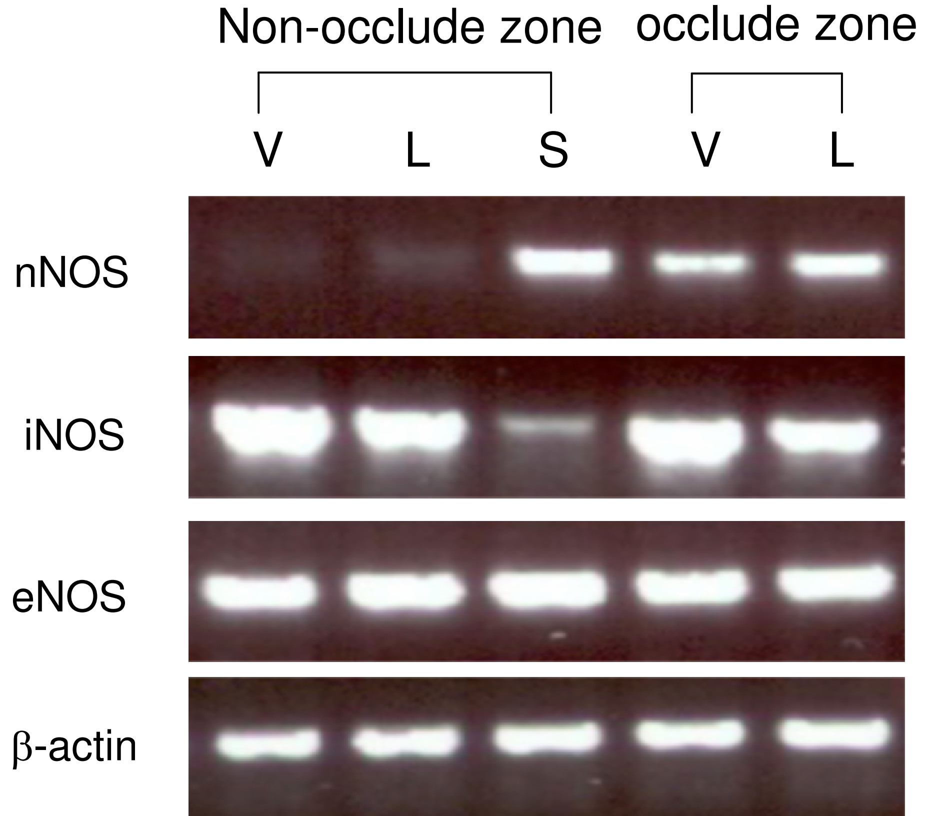

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