Curcumin blocks homocysteine-induced endothelial dysfunction in porcine coronary arteries

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Purpose: **Curcumin, a yellow polyphenolic compound from the plant *Curcuma ionga*, is a commonly used spice and coloring agent with beneficial effects of anti-tumor, anti-inflammatory, and antioxidant activities. The objective of this study was to determine the effect of curcumin on homocysteine-induced endothelial dysfunction in a porcine coronary artery model.**

Methods: **Porcine coronary arteries were cut into 5-mm rings, which were incubated for 24 hours either as control rings, with homocysteine (50 μmol/L), curcumin (5 μmol/L), or a combination of curcumin (5 μmol/L) and homocysteine (50 μmol/L). Myograph tension analysis was performed in response to vessel active drugs including thromboxane A_2 analog U466419 (contraction), endothelium-dependent vasorelaxation (bradykinin), and endothelium-independent vasorelaxation (sodium nitroprusside). Immunohistochemical staining was performed for endothelial nitric oxide synthase (eNOS). In addition, superoxide anion production was determined by lucigenin-enhanced chemiluminescence.**

Results: **All groups of porcine coronary artery rings showed no difference in maximal contraction after U46619 challenge. However, endothelium-dependent vasorelaxation in response to 10^{-5} mol/L bradykinin was 40% in the homocysteine-treated group, as compared to 73% in the control group ($P = .03$). Of importance, curcumin could effectively block homocysteine-induced impairment of endothelium-dependent vasorelaxation. All groups showed no difference in endothelium-independent vasorelaxation. In addition, eNOS immunoreactivity was reduced in the homocysteine group, but the combined homocysteine and curcumin group showed eNOS levels comparable to those in the control group. Furthermore, superoxide anion levels of the endothelial layer were significantly increased by 2-fold in homocysteine-treated vessels as compared to control vessels ($P = .02$), whereas curcumin could block the effect of homocysteine on superoxide anion production.**

Conclusion: **These data demonstrate that curcumin effectively reverses the endothelial dysfunction induced by homocysteine. In addition, curcumin significantly blocked homocysteine-induced superoxide anion production and eNOS down-regulation. This study suggests a therapeutic role for dietary curcumin in patients with homocysteinemia, thereby reducing cardiovascular morbidity and mortality. (J Vasc Surg 2004;40:1216-22.)**

Clinical Relevance: **Hyperhomocysteinemia is a significant clinical problem. It is an independent risk factor for cardiovascular diseases. This study provides new information for better understanding the molecular mechanisms of homocysteine-induced vascular injury. More importantly, curcumin, a natural substance, can effectively block the detrimental effect of homocysteine on the vascular system. Thus curcumin could be used in patients with hyperhomocysteinemia, and to prevent cardiovascular diseases.**

Epidemiologic studies have documented the relationship between risk factors and the development of atherosclerotic disease. Traditional risk factors for atherosclerosis include diabetes, hypertension, hypercholesterolemia, age, and male gender.[1] More recently hyperhomocysteinemia has emerged as an independent risk factor for development of coronary, cerebrovascular, and peripheral arterial occlusive disease.[2] Although severe hyperhomocysteinemia is rare, mild elevations in homocysteine concentration have been found in nearly 7% of the general population and in 20% to 30% of patients with coronary and peripheral vascular disease.[3-5] A mere increase of 12% over the normal level of homocysteine has been associated with a 3-fold increase in risk for myocardial infarction.[6]

Hyperhomocyteinemia exerts its deleterious effect by causing endothelial injury and dysfunction, followed by platelet activation and thrombus formation.[7] Although molecular mechanisms of homocysteine-induced vascular injury are not fully elucidated, increased oxidative stress and decreased nitric oxide (NO) bioavailability have important roles in endothelial dysfunction.[7,8]

Whereas oxidants impair endothelial function, it has been suggested that the use of antioxidants may reverse some of the deleterious effects and improve endothelial

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vasomotor function. Recent studies have highlighted the protective effect of dietary antioxidants on the vascular endothelium.[8-10] Curcumin, a polyphenolic compound obtained from the plant *Curcumin ionga*, is a commonly used spice and coloring agent. Several studies have demonstrated the beneficial effects of curcumin, which include anti-inflammatory,[11] anti-tumor,[12] and antioxidant activity.[13] However, curcumin has not been studied in the vascular system to prevent risk factors such as homocysteine from vascular injury. Thus the aim of this study was to determine whether curcumin could effectively reverse homocysteine-induced endothelial dysfunction, with reference to endothelium-dependent vasorelaxation, endothelial NO synthase (eNOS) expression, and superoxide anion production, in porcine coronary arteries. The findings of this study could suggest a new strategy to prevent or treat homocysteine-induced vascular disease.

METHODS

Reagents. Thromboxane A_2 analog (9,11-dideoxy-11a,9a-poxymethylprostaglandin $F_{2\alpha}$; U46619), homocysteine (DL-homocysteine dissolved in normal saline solution), bradykinin, TRIS-buffered saline solution, phosphate-buffered saline solution (PBS), and curcumin (water soluble, 95% purity), were obtained from Sigma. Curcumin (diferuloylmethane), a single chemical compound, is a major constituent of the plant *Curcumin ionga*. Dulbecco's modified Eagle's medium was obtained from Life Technologies Inc, and antibiotic-antimycotic from Mediatech Inc. Monoclonal antibody against human eNOS was purchased from B. D. Transduction Laboratories. The avidin-biotin complex immunoperoxidase kit was obtained from DAKO, lucigenin was obtained from Molecular Probes, and the horseradish peroxidase–conjugated goat anti-mouse secondary antibodies and the enhanced chemiluminescence kit were from Amersham Life Sciences.

Isometric tension in porcine coronary arteries. The myograph system used in this study has been described.[8,14] Eight pig hearts were harvested from 8-month-old to 10-month-old pigs from a local slaughterhouse. The hearts were then rinsed in cold sterile PBS, the coronary arteries were then perfused with cold PBS, and the hearts were placed in cold PBS for transfer back to the laboratory. The right coronary artery was then carefully dissected out, removing the loose connective tissue, and cut into 5-mm rings, which were then placed in Dulbecco's modified Eagle's medium as control, with homocysteine (50 μmol/L), curcumin (5 μmol/L), or a combination of curcumin (5 μmol/L) and homocysteine (50 μmol/L). A single dose of curcumin (5 μmol/L) was selected on the basis of several previous publications in which the dose showed a potent inhibitory effect on hypoxia-induced oxidative stress[13] or growth factor–induced cell proliferation[15] in human endothelial cells in vitro. The culture medium supplemented with curcumin (5 μmol/L) maintained pH at 7.4. All rings were incubated at 37°C in a 5% CO_2 cell culture incubator. After 24 hours of incubation, the rings were suspended between the wires of an organ bath myograph chamber (Danish Myo Technologies) in 6 mL of Kreb's solution at 37°C and oxygenated with pure oxygen. The rings were subjected stepwise to a predetermined optimal tension of 3*g* and allowed to equilibrate for 30 minutes. After equilibration, each ring was precontracted with thromboxane A_2 analog U46619 (10^{-7} mol/L). Endothelium-dependent vasorelaxation curves were obtained with 5 cumulative additions of 50 μL of endothelium-dependent vasodilator bradykinin (10^{-9}, 10^{-8}, 10^{7}, 10^{-6}, 10^{-5} mol/L) at 3-minute intervals. Endothelium-independent vasorelaxation was assayed with the addition of a single dose of sodium nitroprusside (10^{-6} mol/L). The percentage relaxation was calculated on the basis of tension changes of precontracted vessel rings. The vessel was precontracted with U46619 where the tension value was considered as 0% relaxation. The value of vessel tension before contraction with U46619 (pretension) was considered 100% relaxation. Eight sets of coronary rings were available for each treatment group, and data obtained from several coronary arteries were arranged and represented as 1 data point for statistical analysis.

Immunohistochemistry. The porcine coronary artery rings were fixed overnight in 10% neutral buffered formalin, then transferred to 70% alcohol. Dehydration of the specimen was achieved with sequentially increasing concentrations of ethanol followed by xylene, and embedded in paraffin. Five-micrometer cross sections were then cut, stained with hemotoxylin-eosin, and then stained immunohistochemically for eNOS with the avidin-biotin complex immunoperoxidase procedure. Monoclonal antibody against human eNOS (1:1000) was used.[8]

Detection of superoxide. Coronary arteries were isolated from 3 additional pig hearts. Superoxide levels produced by endothelial cells of the vessel rings were determined with the lucigenin-enhanced chemiluminescence method with a Sirius luminometer and FB 12 software (Berthold Detection System). After 24 hours of culturing, the rings were rinsed briefly in modified Krebs' HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffer solution (120 mmol/L of NaCl, 4.7 mmol/L of KCl, 1.18 mmol/L of K_2HPO_4, 20 mmol/L of HEPES, 2.5 mmol/L of $CaCl_2$, 1.17 mmol/L of $MgSO_4$, and 25 mmol/L of $NaHCO_3$). The rings were then cut open longitudinally into approximately 5 × 5-mm pieces. Assay tubes (12 × 75 mm) were filled with 500 μL of Krebs' HEPES buffer solution and 50 μmol/L of lucigenin. The reagents were gently mixed in the tubes, and the vessel segments were placed with the endothelium side down. Measurements were made every 15 seconds for 12 minutes. The data in relative light units per second (RLU/s) for each sample was averaged between 7 and 10 minutes. Values for blank tubes containing the same reagents as the vessel ring samples were subtracted from the corresponding vessel samples. The area of each vessel segment was measured with a caliper, and used to normalize the data for each sample. The final unit is RLU/s/mm^2.

Statistical analysis. Differences between the groups was analyzed with the 2-tailed Student *t* test for paired data,

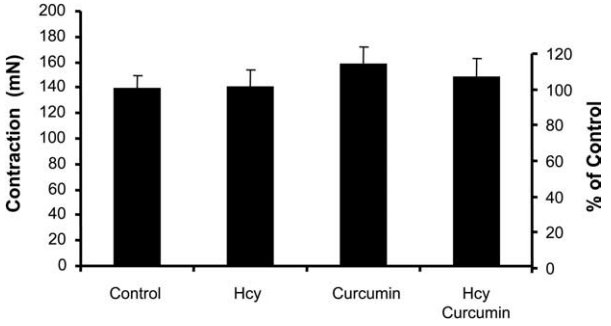

Fig 1. Contraction of porcine coronary arteries. Myograph isometric tension apparatus was used to study maximal contraction of porcine coronary artery rings in response to thromboxane A_2 analog U46619 (10^{-7} mol/L). There was no significant difference among control, homocysteine *(Hcy)*, curcumin, and homocysteine plus curcumin groups (n = 8; $P > .05$, Student *t* test).

with a significance level of $P < .05$ (Microsoft Excel program). Analysis of variance (ANOVA) was used for comparison of vessel response to multiple doses of bradykinin between treated and control vessels. The results are reported as mean ± SE.

RESULTS

Effect of homocysteine and curcumin on vasomotor function. The maximal vessel tension achieved by the addition of thromboxane A_2 analog U46619 (10^{-7} mol/L) showed no difference between groups, including control, homocysteine, curcumin, and homocysteine plus curcumin ($P > .05$, Student *t* test; Fig 1). However, endothelium-dependent vasorelaxation showed a significant difference between groups (Fig 2). In general (Fig 2, *A*), homocysteine significantly impaired endothelium-dependent relaxation in response to multiple doses of bradykinin, as compared with controls (n = 8; $P < .05$, ANOVA). In response to 10^{-5} bradykinin (Fig 2, *B*) the relaxation was only 40% ± 2% in the homocysteine group, as compared to 72% ± 2% in the control group (n = 8; $P < .05$, Student *t* test). Of importance, curcumin had a significant protective effect on vessel wall injury from homocysteine. The rings treated with a combination of homocysteine and curcumin showed a significant increase in vasorelaxation, as compared with homocystein-treated vessels (n = 8; $P < .01$, Student *t* test), which was similar in the control vessels. In addition, curcumin alone did not affect vasorelaxation, as compared with the control group. All groups showed no significant difference in endothelium-independent relaxation, demonstrated by the addition of a single dose of sodium nitroprusside (10^{-6} mol/L; Fig 3).

Effect of homocysteine and curcumin on eNOS immunoreactivity. Homocysteine-treated vessels showed significantly less staining of eNOS protein in comparison with control vessels. In contrast, the homocysteine plus curcumin-treated group showed staining levels of eNOS

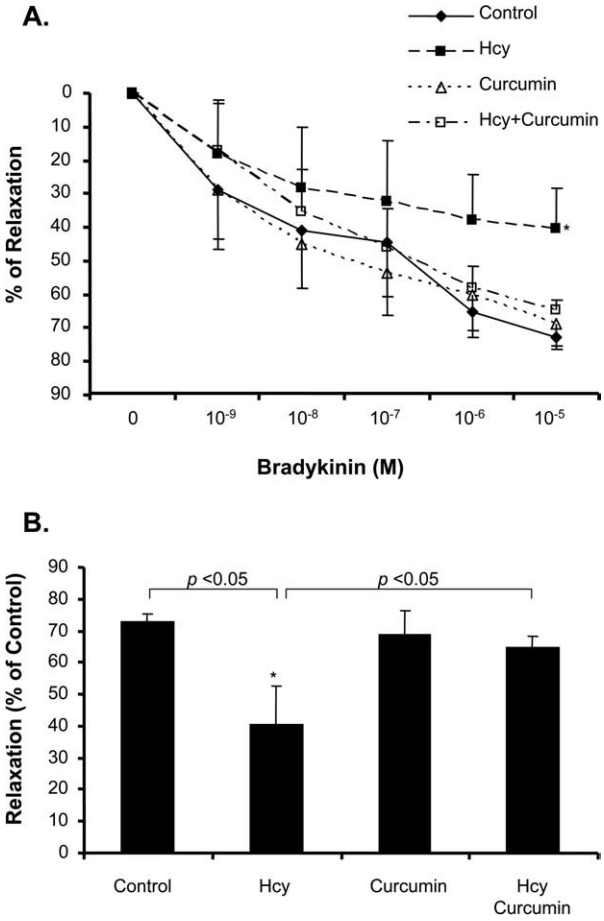

Fig 2. Endothelium-dependent vasorelaxation. **A,** Vasorelaxation in response to various doses of bradykinin. **B,** Vasorelaxation in response to bradykinin at 10^{-5} mol/L. In response to varying strengths of bradykinin, relaxation of precontracted vessel rings was measured with a myograph isometric tension apparatus. Endothelium-dependent vasorelaxation showed a significant difference among groups. Homocysteine *(Hcy)* significantly reduced bradykinin-induced relaxation in a dose-dependent manner, as compared with controls (n = 8; $P < .05$, ANOVA test for **A**, Student *t* test for **B**). Curcumin significantly blocked this effect of homocysteine. Curcumin alone had no effect on vasorelaxation.

protein comparable to that in the control group, indicating the protective effect of curcumin on endothelial cells (Fig 4).

Effect of homocysteine and curcumin on superoxide anion production. The effect of curcumin on homocysteine-induced superoxide anion production is shown in Fig 5. The chemiluminescence of the endothelium showed that control vessels had superoxide levels of 13 ± 2 RLU/s/mm^2; treatment with homocysteine led to a 2-fold increase in superoxide levels to 40 ± 8 RLU/s/mm^2 (n = 3; $P < .05$, Student *t* test), whereas the combined homocysteine and curcumin group had a reduced level of only 10 ± 1 RLU/s/mm^2.

DISCUSSION

A major finding of this study was that curcumin effectively reversed the endothelial dysfunction induced by homocysteine in a porcine coronary artery model. Treatment of porcine coronary artery rings with 50 μmol/L of homocysteine showed a significant reduction of endothelium-dependent relaxation in response to bradykinin. In addition, homocysteine reduced eNOS levels and increased superoxide production. Of importance, the incubation of the vessel rings with homocysteine plus curcumin effectively reversed the deleterious effects of homocysteine. Treatment with curcumin restored the endothelium-dependent relaxation, increased eNOS protein levels, and decreased superoxide production.

Curcumin, a commonly used spice and coloring agent, has recently been shown to have a spectrum of beneficial effects, ranging from anti-inflammatory,[11] anti-tumor,[12] and antioxidant activity.[13] The anti-inflammatory action is in part due to the free radical scavenging property of curcumin, which in turn leads to suppression of superoxide production by macrophages, in addition to countering the effects of lipopolysaccharide-induced activation of tumor necrosis factor-α and interleukin-1. A recent study showed that curcumin pretreatment to endotoxemic mice effectively reduced the phagocytic activity of Kupffer cells and also endothelial swelling.[11] It has also been suggested that the anti-inflammatory action of curcumin may be attributable to inhibition of leukocyte recruitment[16] or inhibition of tumor necrosis factor-α–induced expression of adhesion molecules.[17] Curcumin inhibits angiogenic differentiation of human umbilical vein cells by possible modulation of protease activity during endothelial morphogenesis.[12] Other postulated anti-angiogenic mechanisms of curcumin involve the inhibition of activity of thymidine kinase enzyme, which effectively blocks cell cycle progression and up-regulation of cyclin-dependant kinase inhibitors.[15] The protective action of curcumin on the vascular endothelium against oxidative stress has also been reported. One study suggested that a possible mechanism may involve the production of endothelial heme-oxygenase, an inducible stress protein that degrades heme to the vasoactive molecule and the antioxidant biliverdin.[13] Furthermore, curcumin protects endothelial cells from the oxidative stress induced by β-amyloid, implicated as a well-established pathway inducing neuronal cell death in Alzheimer's disease.[18]

Despite extensive literature on the anti-inflammatory, anti-tumor, and antioxidant activity of curcumin, we are not aware of any studies that have documented curcumin reversal of homocysteine-induced endothelial dysfunction. Our study showed that curcumin can effectively block homocysteine-induced decrease in endothelium-dependent vasorelaxation, with a concentration of curcumin as low as 5 μmol/L. This assay was the measure of the vessel response to bradykinin. It is unlikely that curcumin could interact with the bradykinin receptor on endothelial cells because vessels treated with curcumin alone did not affect

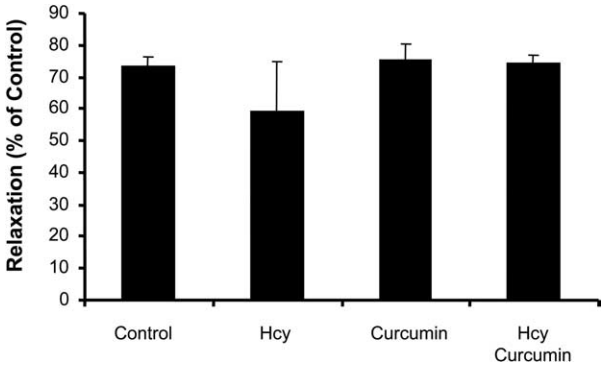

Fig 3. Endothelium-independent vasorelaxation. In response to a single dose of sodium nitroprusside (10^{-6} mol/L), relaxation of porcine coronary rings was measured. All groups showed no significant difference in endothelium-independent relaxation (n = 8; $P < .05$, Student *t* test).

the vessel response to bradykinin, as compared with the control vessels. EC_{50} for bradykinin is the effective concentration of bradykinin that induces 50% maximum relaxation and is usually used to express the sensitivity of the tested vessel to bradykinin.[19,20] In our study the maximum relaxation among groups was different because of different treatment conditions. Thus EC_{50} for bradykinin would not be a sensitive measure for our data. Rather, the maximun relaxation in response to bradykinin (10^{-5} mol/L) between groups (Fig 2, *B*; paired Student *t* test) or ANOVA for multiple data points (Fig 2, *A*) among groups would be the best methods to present our data. Recent studies have used curcumin in doses up to 80 mg/kg with no reported side effects.[11] It is possible that curcumin confers its beneficial and protective endothelial effect by decreasing superoxide levels. Indeed, our results demonstrate that curcumin significantly reduced superoxide anion production in porcine coronary arteries. This would be similar to the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors.[21] The results of this study are comparable to those of a recent study that used a dietary antioxidant, red wine, to reverse homocysteine-induced endothelial dysfunction in porcine coronary arteries.[8] However, a dose-dependent study of curcumin or homocysteine is not included in the study, which could be a limitation. Further investigation of potential applications of curcumin in vascular systems, including a broad dose range, animal models, and human trials are warranted.

NO produced from eNOS is a known regulator of vascular tone, blood pressure, and antithrombotic activity.[22-24] Of importance, inadequate NO production and decreased eNOS expression are seen in atherosclerosis and other vascular injury.[25] Homocysteine also increases potent reactive oxygen free radicals, and this may also explain the decreased availability of NO, because superoxide anion is readily reacted with NO to form peroxynitrite anion.[26] In our study homocysteine-treated porcine rings also showed a decrease in eNOS immunoreactivity in comparison with

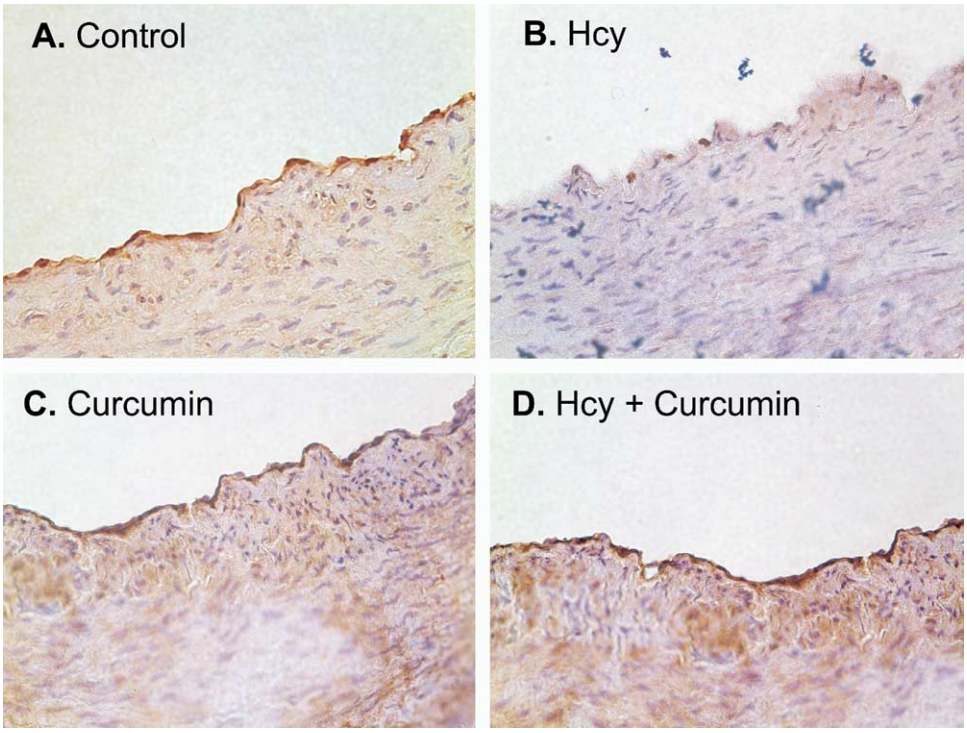

Fig 4. Immunohistochemical staining of endothelial nitric oxide synthase (eNOS). Porcine coronary artery rings were cultured in different groups for 24 hours. eNOS levels were stained with the avidin-biotin complex immunoperoxidase procedure. Homocysteine *(Hcy)*–treated vessels showed less staining of eNOS protein in comparison with control vessels. In contrast, the homocysteine plus curcumin-treated group showed a staining level of eNOS protein comparable to the control group. **A,** Control group. **B,** Homocysteine (50 μmol/L) treatment. **C,** Curcumin (5 μmol/L) treatment. **D,** Homocysteine (50 μmol/L) plus curcumin (5 μmol/L) treatment. (Original magnification ×400.)

control rings. Thecombination of curcumin and homocysteine led to eNOS staining levels comparable to control porcine rings. However, quantitative measurement of the eNOS expression, including both messenger RNA and protein levels, was not performed in this study and is warranted for further investigation.

A single dose of curcumin (5 μmol/L) was used in our in vitro study of porcine coronary arteries. It is not known whether this concentration is attainable with dietary supplementation in human beings. Curcumin or extract of *Curcumin longa* has been used in human trials. However, the pharmacokinetics of curcumin in human beings is unknown as yet. Healthy human volunteers received a daily oral dose of 200 mg of curcumin extract (ZCL4 curcuma) for 40 or 60 days and demonstrated cardiovascular benefits such as serum lipid peroxides but absence of any detectable toxicity.[27,28] In other reports individuals who received a daily oral dose of 1200 to 2500 mg of curcumin for various periods of time exhibited lack of toxicities.[29-33] However, the plasma level of curcumin was not measured in these reports. In a clinical pilot study oral doses of 4 to 8 g of curcumin for 3 months generated plasma levels of 0.51 to 1.77 μmol/L.[34] However, curcumin concentrations in the tissues, including blood vessels, have not been studied in human trials. Thus our in vitro dose of curcumin (5 μmol/L) is higher than clinical plasma levels. Effects of a dose of less than 5 μmol/L of curcumin will be interesting to include in the future investigation of our model system, to show clinical relevance. In addition, there is no information about the effect of curcumin in patients with hyperhomocysteinemia. However, curmumin did show antioxidant effect in human trials.[27,28] Inasmuch as a large amount of data, including those in this study, show that homocysteine can induce vascular injury through oxidative stress, curcumin could logically be effective to block the detrimental effects of homocysteine in patients with hyperhomocysteinemia. Further clinical investigation is warranted in this regard.

In summary, homocysteine impairs endothelium-dependent vasorelaxation, decreases eNOS levels, and increases superoxide anion levels, and curcumin can block all of these effects of homoncysteine in the porcine coronary artery culture model. The mechanism of the inhibitory effect of curcumin on homocysteine-induced vessel injury may involve the antioxidant function of curcumin. Other mechanisms such as direct interaction between curumin and homocysteine may exist and warrant further investigation.

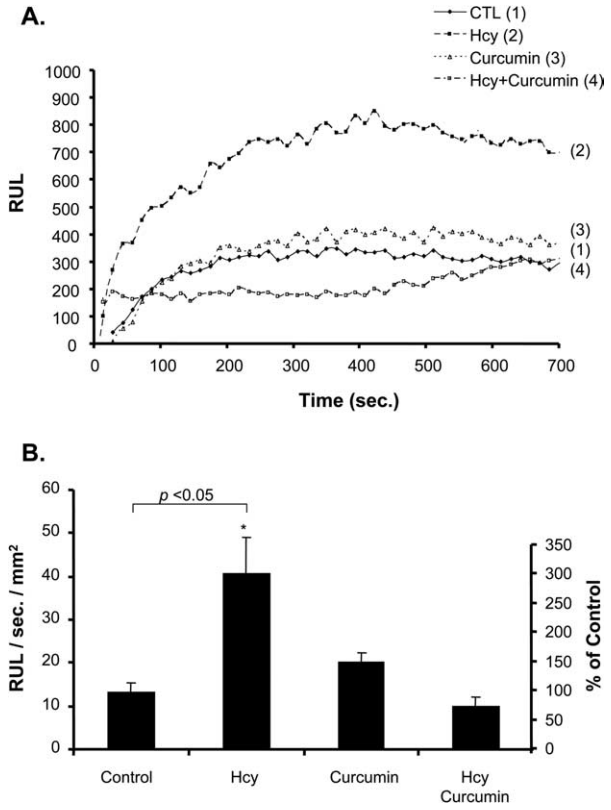

Fig 5. Superoxide anion production. Superoxide anion levels of endothelial layer of cultured porcine coronary rings were determined with the lucigenin-enhanced chemiluminescence method. Homocysteine-treated rings showed a significantly high level of superoxide anion, as compared with control rings ($n = 3$; $P < .05$, Student *t* test), whereas the homocysteine plus curcumin group demonstrated a reduced level of superoxide anion, less than that in the control group. **A,** Kinetic recording of chemiluminescence in typical set of experiments. **B,** Normalized superoxide anion levels in vessel rings. *CTL,* Control; *Hcy,* homocysteine.

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