Effects of Hypertension, Diabetes Mellitus, and Hypercholesterolemia on Endothelin Type B Receptor–Mediated Nitric Oxide Release From Rat Kidney

Masao Kakoki, MD; Yasunobu Hirata, MD; Hiroshi Hayakawa, MD; Akihiro Tojo, MD; Daisuke Nagata, MD; Etsu Suzuki, MD; Kenjiro Kimura, MD; Atsuo Goto, MD; Kazuya Kikuchi, PhD; Tetsuo Nagano, PhD; Masao Omata, MD

Background—Although endothelin-1 is a potent vasoconstrictor peptide, stimulation of endothelin type B receptor (ETBR) causes bidirectional changes in vascular tone, ie, vasodilation and vasoconstriction. Roles of ETBR in pathological conditions are largely unknown.

Methods and Results—We studied the effect of BQ-3020, a highly selective ETBR agonist, on renal vascular resistance and nitric oxide (NO) release in the isolated, perfused kidney of rats with hypertension, diabetes mellitus, and hypercholesterolemia. Immunohistochemistry of endothelial NO synthase and ETBR was also examined. Infusion of BQ-3020 at concentrations of \(10^{-10}\) mol/L reduced renal perfusion pressure in Dahl salt-resistant (R) rats but increased renal perfusion pressure in Dahl salt-sensitive (S) rats (\(10^{-10}\) mol/L: -10.3±0.6% versus 11.2±1.5%, R versus S; \(P<0.01\)). BQ-3020 caused a dose-dependent release of NO in both R and S rats, although the level of NO release in S rats was lower, as detected by chemiluminescence (\(10^{-10}\) mol/L: 10.7±0.7 versus 3.1±0.4 fmol/min per gram of kidney, R versus S; \(P<0.01\)). Similar effects of BQ-3020 were observed in streptozotocin-induced diabetic rats and diet-induced hypercholesterolemic rats. Expression of endothelial NO synthase decreased in S rats but not in diabetic or hypercholesterolemic rats. In contrast, expression of ETBR in the endothelium was decreased in all 3 disease models compared with that in the vascular smooth muscle cell.

Conclusions—These results suggest that impaired NO release in response to stimulation of ETBR is due, at least in part, to a decrease in endothelial ETBR and may play a role in vascular dysfunction usually associated with arteriosclerosis-related diseases. (Circulation. 1999;99:1242-1248.)

Key Words: immunohistochemistry ■ diabetes mellitus ■ hypertension ■ hypercholesterolemia ■ angiotensin

Endothelin is a potent vasoconstrictor peptide discovered in cultured porcine endothelial cells.1 Of the 3 endothelin isopeptides known, endothelin 1 (ET-1) is the only peptide produced in the vascular endothelium. ET-1 exerts its effects via 2 subtypes of endothelin receptor in mammals, endothelin A receptor (ETAR)2 and endothelin type B receptor (ETBR).3 Both receptors are present on vascular smooth muscle cells (VSMCs) and mediate vasoconstriction.4 However, only ETBR is present on vascular endothelial cells, and its stimulation induces vasodilation through the release of nitric oxide (NO).5 Thus, ET-1 can have bidirectional effects on vascular tone.

It is unclear what role ET-1 plays in the control of vascular tone in the normal condition. Recently, it was shown that mice deficient in the ET-1 gene had higher blood pressure than wild-type mice, which suggests a hypotensive effect of ET-1.6 However, other studies7,8 using anti–ET-1 antibodies and endothelin receptor antagonists have revealed that ET-1 does not play a major role in the maintenance of normal hemodynamics.

In pathophysiological conditions with endothelial dysfunction, expression of ET-1 is increased and vasoconstriction in response to ET-1 is enhanced; these phenomena suggest that ET-1 contributes to regulation of vascular tone. However, the hypotensive effects of ETAR antagonists are not consistent. BQ-123, a potent ETAR antagonist, does not reduce blood pressure in spontaneously hypertensive rat (SHR). On the other hand, antagonists that block both ETAR and ETBR seem to be more effective in reducing blood pressure than those that block only ETAR, at least in SHR.11 These results suggest that ETBR plays a role in blood pressure control.

We have reported the attenuated response of deoxycorticosterone acetate (DOCA)–salt rat kidney to ETBR stimul-
tion with respect to NO release and renal vasorelaxation.\textsuperscript{12} We suggested the downregulation of ETBR in the DOCA-salt kidney as its mechanism because ETBR mRNA was reduced in the whole kidney of DOCA-salt rats. However, it is unclear whether this altered ETBR mRNA reflected that of the endothelial cells and whether this is commonly the case in other types of hypertension, such as Dahl rats and SHR. Furthermore, because hypercholesterolemia and diabetes mellitus (DM) as well as hypertension are major risk factors for cardiovascular diseases, it is important to know whether similar endothelial alterations in ETBR exist in these states.

In the present study, we investigated the effects of ETBR stimulation on NO release and vascular tone in renal vessels in rats with hypertension, DM, or hypercholesterolemia by infusing the isolated, perfused kidney with BQ-3020, a highly selective ETBR agonist. We also examined immunohistochemistry of endothelial NO synthase (eNOS) and ETBR to explore the mechanism(s) responsible for decreased NO release in these renal vessels.

**Methods**

**Animals**

Six-week-old male Dahl salt-sensitive (S) and Dahl salt-resistant (R) rats (Eizai Co., Japan) were fed high-salt (8%) rat chow for 8 weeks. Eight-week-old male Wistar-Kyoto rats (WKY) and SHR (Charles River Japan) were administered streptozotocin (STZ; 35 mg/kg i.v.) to induce diabetes 4 weeks before the experiment. In an experiment to examine the effects of an ACE inhibitor, some WKY and SHR with DM were administered imidapril (10 mg/d SC) by use of an osmotic minipump (Alzet 2 ML4, Alza Co) for 4 weeks. High-cholesterol chow (4%) containing 1% cholic acid was fed to 8-week-old Sprague-Dawley rats for 12 weeks to induce hypercholesterolemia. Each group of rats was compared with age-matched controls.

Systolic blood pressure was measured in unanesthetized rats by the tail-cuff method. After an overnight fast, blood was drawn for determination of plasma glucose and cholesterol. The rats were treated according to the “Guide for Animal Experimentation” of the Faculty of Medicine, University of Tokyo, Japan.

**Isolated, Perfused Kidney and Measurement of NO Release**

Rats were anesthetized with pentobarbital sodium (40 mg/kg IP). The kidneys were isolated and perfused as described elsewhere.\textsuperscript{12,13} Kidneys were perfused with Krebs-Henseleit buffer containing 10$^{-2}$ mol/L phenylephrine and 10$^{-2}$ mol/L indomethacin at a constant flow rate of 5 mL/min. NO in the effluent perfusate from the renal vein was measured with a chemiluminescence detector (825-CL, Jasco Corp), as previously described.\textsuperscript{13}

**Immunohistochemistry**

Immunohistochemical staining of the renal tissue for eNOS and ETBR was performed as described previously.\textsuperscript{14} Wax sections were labeled with primary antisera raised against eNOS (mouse monoclonal antibody; Transduction Laboratories) and ETBR\textsuperscript{15} (rabbit polyclonal antibody, Immunobiological Laboratories), stained according to the streptavidin-biotin method.

Intensity of staining was graded from 0 to 2: 0=no staining, 1=slight staining, and 2=moderate or intense staining. Scoring was performed in a blinded manner by one of the investigators (A.T.). Fifteen arteries from each animal kidney (n=4 per group) were scored and averaged. Averaged scores were compared statistically.

For ETBR, the degree of endothelial cytosolic stainings was compared with that in VSMCs.

**Experimental Protocol**

After a 60-minute equilibration period, the effects of vehicle, BQ-3020 (Calbiochem), and $\text{N}^\text{G}$-nitro-l-arginine (L-NNA) on renal perfusion pressure (RPP) and NO release were measured in rats with hypertension, DM, and hypercholesterolemia in and control rats. In rats with hypercholesterolemia and their control rats (n=4 per group), effects of ET-1 were also studied. Drugs were infused in the following order at 10-minute intervals: vehicle, then 10$^{-12}$, 10$^{-11}$, 10$^{-10}$, 10$^{-9}$, and 10$^{-8}$ mol/L BQ-3020; or vehicle, then 10$^{-12}$, 10$^{-11}$, 10$^{-10}$, 10$^{-9}$, and 10$^{-8}$ mol/L ET-1. Thereafter, 10$^{-8}$ mol/L L-NNA was added to 10$^{-8}$ mol/L BQ-3020. To confirm the receptor specificity of BQ-3020, we also examined its effect in WKY in the presence or absence of 10$^{-6}$ mol/L BQ-123 and 10$^{-8}$ mol/L BQ-788, selective ETAR and ETBR antagonists, respectively. Plasma ET-1 concentration was determined by radioimmunoassay.\textsuperscript{16}

**Statistical Analysis**

Values are expressed as mean±SEM. ANOVA was used to analyze continuous variables. Scheffé modified t test was then used for multiple comparisons. Mann-Whitney U test or Kruskal-Wallis test was used to compare the intensity of immunostaining when appropriate. The level of significance was taken at P<0.05.

**Results**

**Effects of BQ-3020 and Plasma ET-1 in Dahl Rats**

Systolic blood pressure, kidney weight, and kidney to body weight ratio were significantly greater in S rats than in R rats (Table). As shown in Figure 1, BQ-3020 at doses of $\leq$10$^{-10}$ mol/L caused vasorelaxation in R rats, whereas it caused vasoconstriction in S rats. BQ-3020 at doses of $\leq$10$^{-9}$ mol/L induced an increase in RPP in both R and S rats, but the extent of RPP elevation was significantly greater in S rats than in R rats. BQ-3020–induced NO release at any concentration was less in S rats than in R rats. Dahl S rats had significantly higher levels of blood pressure and plasma ET-1 than Dahl R rats (blood pressure 202±21 versus 138±12 mm Hg, P<0.001; ET-1 4.0±0.2 versus 2.5±0.3 pg/mL, P<0.05).

**Effects of BQ-3020 in Diabetic Rats**

Although nondiabetic SHR had higher systolic blood pressure than nondiabetic WKY (Table), the responses of RPP and NO release to BQ-3020 in SHR were comparable to those in WKY (Figure 2).

Diabetic WKY and SHR had smaller body weights, higher levels of fasting blood glucose, and greater kidney weights than control rats (Table). However, STZ did not alter systolic blood pressure in SHR or WKY. The amount of NO released in response to infusion of BQ-3020 in isolated kidneys in both diabetic WKY and diabetic SHR was significantly smaller than in the respective controls. Subcutaneous administration of imidapril for 4 weeks reduced systolic blood pressure and the heart weight to body weight ratio (data not shown) of both diabetic rat models, whereas it did not affect fasting blood glucose in either diabetic rat model. BQ-3020 at doses of $\leq$10$^{-10}$ mol/L induced vasorelaxation in control rats, whereas it induced vasoconstriction in rats with DM. The extent of BQ-3020–induced RPP elevation at doses of $\geq$10$^{-9}$ mol/L was significantly greater in rats with DM than in control rats. BQ-3020–induced NO release was less in rats with DM than in control rats (Figure 2). These changes in vascular response to BQ-3020 tended to be greater in diabetic...
SHR than in diabetic WKY. Because ACE inhibitors have been shown to protect the endothelial function and to be effective for diabetic nephropathy, we examined the effects of imidapril on ETBR-induced NO release in diabetic rats. Administration of imidapril markedly improved attenuated responses of vasorelaxation and NO release to BQ-3020 in both diabetic WKY and diabetic SHR.

Effects of BQ-3020 and ET-1 in Hypercholesterolemic Rats
Sprague-Dawley rats fed a high-cholesterol diet showed higher serum cholesterol levels (7.8±0.2 versus 1.8±0.1 mmol/L; P<0.01), but they showed comparable systolic blood pressure (Table). BQ-3020 at doses of 10^{-10} mol/L induced vasorelaxation in control rats, whereas it caused vasoconstriction in rats with hypercholesterolemia. As shown in Figure 3A, NO release by BQ-3020 was less in rats with hypercholesterolemia than in control rats.

In rats with hypercholesterolemia, the effects of ET-1 were also assessed. ET-1 at doses of 10^{-11} mol/L induced vasorelaxation in control rats, whereas it caused vasoconstriction in rats with hypercholesterolemia. As shown in Figure 3B, NO release by ET-1 was also less in rats with hypercholesterolemia than in control rats.

Confirmation of BQ-3020 as a Selective ETBR Agonist
In WKY, BQ-123 (10^{-6} mol/L), a selective ETAR antagonist, did not alter the effects of BQ-3020 on RPP or NO release. In contrast, pretreatment with BQ-788 (10^{-8} mol/L), a selective ETBR antagonist, abolished not only BQ-3020–induced vasodilation but also vasoconstriction and markedly inhibited BQ-3020–induced NO release (Figure 4).

Immunohistochemistry of eNOS and ETBR in Hypertensive, Diabetic, and Hypercholesterolemic Rats
We investigated the immunohistochemistry of eNOS and ETBR to examine the mechanism(s) by which the effects of

<table>
<thead>
<tr>
<th>Body Weight, Kidney Weight, Kidney to Body Weight Ratio, Systolic Blood Pressure, and Fasting Blood Glucose in All Rats Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Rats</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>R</td>
</tr>
<tr>
<td>S</td>
</tr>
<tr>
<td>WKY</td>
</tr>
<tr>
<td>WKY-STZ</td>
</tr>
<tr>
<td>WKY-STZ-IMD</td>
</tr>
<tr>
<td>SHR</td>
</tr>
<tr>
<td>SHR-STZ</td>
</tr>
<tr>
<td>SHR-STZ-IMD</td>
</tr>
<tr>
<td>NC</td>
</tr>
<tr>
<td>HC</td>
</tr>
</tbody>
</table>

BW indicates body weight; KW, kidney weight; KW/BW, ratio of kidney weight to body weight; SBP, systolic blood pressure; FBG, fasting blood glucose; IMD, imidapril; NC, normocholesterolemic rats; and HC, hypercholesterolemic rats.

Values are mean±SEM.

*P<0.05 vs control group; †P<0.05 vs STZ (diabetic) group.

Figure 1. Effects of BQ-3020, an ETBR agonist, on RPP and NO release in S and R rats. *P<0.05, †P<0.01 vs R rats.

Figure 2. Effects of BQ-3020 on RPP and NO release in diabetic WKY and SHR. IMD indicates imidapril. *P<0.05, †P<0.01 vs control group; ‡P<0.05, §P<0.01 vs diabetic rats without imidapril.
BQ-3020 were altered in vessels of the 3 disease models. As shown in Figure 5A, immunostaining of eNOS was markedly less in S rats than in R rats. The intensity of eNOS immunostaining was significantly less in S rats than in R rats (S 1.2±0.2 versus R 1.8±0.2; \( P < 0.05 \)). In diabetic and hypercholesterolemic rats, immunostaining of eNOS was comparable to that of the respective controls (data not shown).

Immunostaining of ETBR was observed in both endothelial cells and VSMCs of the renal artery. Figure 5 shows immunostaining of ETBR in renal arteries of the 3 disease models. In S rats, diabetic rats, and hypercholesterolemic rats, less staining of ETBR was observed in the endothelium than was observed in VSMCs, whereas staining of ETBR in the endothelium was commonly greater than that in VSMCs in the respective control groups. Imidapril restored expression of ETBR in the endothelium in diabetic rats. Figure 6 shows the proportion of arterial sections in which intensity of immunostaining of ETBR in the endothelium was greater than that in VSMCs. Relative decreases in the immunostaining of endothelial ETBR were found in S rats, diabetic rats, and hypercholesterolemic rats. Scores of the endothelial ETBR intensity were also significantly lower in these rat groups, whereas those of VSMC intensity were not different between disease models and their controls (data not shown).

**Discussion**

Although acetylcholine has conventionally been used to estimate endothelial function, it is unlikely that it is a major regulator of basal vascular tone and NO release in vivo. In contrast, ET-1 has been shown to be a potent stimulator of eNOS even at picomolar doses.\(^{12}\) We have reported that ETBR stimulation by BQ-3020, particularly at lower doses, more precisely reflects endothelial function than does ET-1, which also stimulates ETAR on VSMCs.\(^{12}\) However, we further examined the effects of ET-1 in rats with hypercholesterolemia and their controls. ET-1 exerted a vasodilatory effect at lower doses but a vasoconstrictive effect at higher doses and a dose-dependent NO release in the kidneys of control rats but only vasoconstrictive effects in the kidneys of hypercholesterolemic rats. In the previous study, we demonstrated that ET-1 had similar effects in the kidneys of DOCA-salt hypertensive rats. We therefore speculate that the effects of BQ-3020 on vascular tone and NO release, especially at physiologically low doses, may be similar to those of ET-1, an endogenous ligand.

To investigate the underlying mechanism(s) for decreased NO release caused by BQ-3020, we examined the immunohistochemistry of eNOS and ETBR in hypertensive, diabetic, or hypercholesterolemic rats. We previously showed that decreased expression of eNOS was associated with decreased NO release in response to acetylcholine, BQ-3020, vasopressin, and adrenomedullin in DOCA-salt hypertensive rats.\(^{12,18,19}\) Similarly, decreased expression of eNOS was observed in S rats. However, no apparent alteration in the expression of eNOS was observed in rats with DM or hypercholesterolemia, which indicates that the impaired NO release in response to ETBR stimulation in DM and hypercholesterolemia cannot be explained by only a decrease in eNOS.

Although it was not clear from the results of the current study why eNOS was reduced in Dahl S rats but not in diabetic or hypercholesterolemic rats, it is possible that the degree of endothelial damage may have been related to the decrease in eNOS. A recent study\(^{20}\) using hypercholesterolemic animals showed that eNOS immunoreactivity in coronary arteries was correlated with areas of intimal proliferation. Because morphological changes were mild in diabetic or hypercholesterolemic rats, this may explain the lack of a decrease in eNOS in these animals. A recent study\(^{20}\) using hypercholesterolemic animals showed that eNOS immunoreactivity in coronary arteries was correlated with areas of intimal proliferation. Because morphological changes were mild in diabetic or hypercholesterolemic rats, this may explain the lack of a decrease in eNOS in these animals. However, in these rats, BQ-3020–induced NO release was decreased. In addition to the downregulation of ETBRs, activation of eNOS or the action of NO in DM and hypercholesterolemia may be impaired. It has been suggested that increases in oxidized LDL in hypercholesterolemia\(^{21}\) and increases in advanced glycosylation end products\(^{22}\) or decreases in NADPH in DM\(^{23}\) may reduce endothelium-dependent vasorelaxation.
A new finding of our present study is that there is markedly less ETBR on renovascular endothelium in rats with hypertension, DM, or hypercholesterolemia than on VSMCs. Thus, the primary cause for vasoconstriction induced by BQ-3020 may be a reduction in endothelial ETBR in the 3 disease models. In contrast to eNOS expression, the decline in ETBR was present in the endothelium regardless of the presence of morphological abnormalities. A decrease in ETBR may be due to an increase in ET-1, which causes receptor downregulation via activation of protein kinase C and intracellular calcium mobilization, because vascular production and plasma levels of ET-1 in animals with DM or hypercholesterolemia have been shown to be elevated. Because plasma levels of ET-1 in Dahl rats have not been fully studied, we measured them and found that ET-1 in S rats was increased compared with R rats. In contrast, plasma levels of ET-1 in SHR were comparable to those in WKY in our previous study, and endothelial ETBR was also comparable to that in WKY in our present study, which suggests that the increase in plasma levels of ET-1 and the decrease in endothelial ETBR are indicators of, or play a causative role in, endothelial damage.

The decrease in NO release in response to ETBR stimulation in Dahl S rats fed a high-salt diet in the present study was similar to that observed in DOCA-salt hypertensive rats in our previous study. However, this finding was not common to all hypertensive animals, because NO release in 12-week-old SHR, whose systolic blood pressure was markedly elevated, was not impaired compared with 12-week-old WKY in the present study. We have previously shown that plasma ET-1 in SHR was not different from that in WKY.

Figure 5. Immunostaining of endothelial NO synthase (A, B) and ETBR (C through I) in the renal arteries. A and C, Dahl R rat. B and D, Dahl S rat. E, Control SHR. F, Diabetic SHR. G, Diabetic SHR treated with imidapril. H, Normocholesterolemic rat. I, Hypercholesterolemic rat.

Figure 6. Proportion of renal arterial sections in which intensity of immunostaining of ETBR in endothelium is greater than that in VSMCs. IMD indicates imidapril; NC, normocholesterolemic rat; and HC, hypercholesterolemic rat. *P<0.05 vs control group; †P<0.05 vs diabetic rats without imidapril.
and expression of endothelial ETBRs in SHR was similar to that in WKY in the present study, which suggests a genetically determined heterogeneous endothelial function in hypertensive animals.

It is striking that imidapril restored the reduced expression of endothelial ETBR in diabetic rats despite no reduction in blood glucose and a slight decrease in systolic blood pressure. The systemic renin-angiotensin system has been considered to be normal or suppressed in DM. However, tissue-specific activation of the renin-angiotensin system in the heart and kidneys of STZ-induced diabetic rats \(^{26,27}\) has been suggested. It has been shown that angiotensin II increases production of ET-1 in various conditions. \(^{28}\) This suggests that angiotensin II mediates the decreased ETBR expression in the endothelium. Therefore, suppression of the renin-angiotensin system may result in restoration of downregulation of ETBR. It seems possible that the beneficial effect of ACE inhibitors in diabetic nephropathy is due in part to restoration of endothelial ETBR suppressed through the locally activated renin-angiotensin system in DM in addition to the preferential dilatation of the renal efferent arterioles to reduce intraglomerular pressure. \(^{17}\)

We provide evidence that an ACE inhibitor (imidapril) was effective in improving endothelial function in diabetic rats in the current study. It has been shown that perindopril, another ACE inhibitor, improved endothelium-dependent vasorelaxation without affecting blood pressure in Dahl S rats. \(^{29}\) Captopril also improved endothelial function without affecting plasma lipids in hypercholesterolemic minipigs, \(^{30}\) which suggests that ACE inhibitors are beneficial to endothelial injury. These findings suggest the existence of a common mechanism in endothelial damage, in which angiotensin II may be involved.

We semiquantified the expression of ETBRs and eNOS using an immunohistochemical method. In a recent study \(^{20}\) using hypercholesterolemic porcine coronary arteries, a scoring system of immunoreactivity for eNOS was adopted that was similar to ours. The monoclonal antibody for eNOS and the polyclonal antibody for ETBR used in the present study have been shown to be highly specific to their antigens, as determined by immunoblot analysis in previous studies. \(^{15}\) Furthermore, this scoring method revealed that in most control vessels, the density of endothelial ETBRs was much greater than that of medial ETBRs, which suggests that this immunohistochemical method may at least be useful to evaluate marked alterations of ETBRs and eNOS.

Recently, it has been demonstrated that endothelin antagonists mitigate tissue damage in various diseases, including arteriosclerosis. \(^{11,13,31}\) However, it is not clear which ETAR antagonists or ETAR/ETBR antagonists are more effective in such diseases, because it is controversial whether blocking ETBR is beneficial or not. The present study suggests that ETAR/ETBR antagonists are more useful than ETAR antagonists in improving peripheral circulatory disturbances in hypertension, DM, and hypercholesterolemia because ETBR stimulation rarely causes vasodilation at any dose.

In conclusion, NO release in response to stimulation of ETBR was reduced in renal vessels in hypertension, DM, and hypercholesterolemia, in part because of a decrease in endothelial ETBR. This may play a role in the vascular dysfunction that is usually associated with arteriosclerosis-related diseases.

**Acknowledgments**

This study was supported in part by grants-in-aid No. 07557055 and No. 09281206 from the Japanese Ministry of Education, Culture, and Science. The authors wish to thank Tanabe Pharmaceutical Co for their generous gift of imidapril.

**References**


Effects of Hypertension, Diabetes Mellitus, and Hypercholesterolemia on Endothelin Type B Receptor–Mediated Nitric Oxide Release From Rat Kidney

Circulation. 1999;99:1242-1248
doi: 10.1161/01.CIR.99.9.1242

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/99/9/1242

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/