Lipid Raft Clustering and Redox Signaling Platform
Formation in Coronary Arterial Endothelial Cells

Andrew Y. Zhang, Fan Yi, Guo Zhang, Erich Gulbins, Pin-Lan Li

Abstract—Recent studies have indicated that lipid rafts (LRs) in the cell membrane are clustered in response to different stimuli to form signaling platforms for transmembrane transduction. It remains unknown whether this LR clustering participates in redox signaling in endothelial cells. The present study tested a hypothesis that clustering of LRs on the membrane of coronary endothelial cells produces aggregation and activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, thereby forming a redox signaling platform. By confocal microscopic analysis of agonist-stimulated rafts patch formation, we found that several death receptor ligands or apoptotic factors, including tumor necrosis factor α, Fas ligand, or endostatin, stimulated the clustering and trafficking of individual LRs on the plasma membrane of coronary endothelial cells. Interestingly, double labeling of a membrane-bound NADPH oxidase subunit, gp91phox, and LRs showed that gp91phox colocalized within the LR patches when endothelial cells were stimulated by Fas ligand. In isolated LR fractions from Fas-stimulated endothelial cells, gp91phox, p47phox (a crucial cytosolic regulatory subunit of NADPH oxidase), and Rac GTPase were markedly increased and blocked by nystatin, a compound that disrupts LRs. These clustered LRs contained high NADPH oxidase activity, which increased in response to Fas stimulation. Functionally, Fas ligand–induced inhibition of endothelium-dependent vasorelaxation was reduced if LRs were disrupted or NADPH oxidase was inhibited. These results suggest that LR clustering occurs in coronary endothelial cells. The formation of redox signaling platforms on the cell membrane mediates transmembrane signaling of death receptors, resulting in endothelial dysfunction. (Hypertension. 2006;47:74-80.)

Key Words: lipids ● signal transduction ● endothelium ● free radicals

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ernently, lipid rafts (LRs) on the cell membrane have been demonstrated to be clustered or aggregated in response to different stimuli and thereby to play an important role in transmembrane signaling in a variety of mammalian cells.1,2 LRs consist of dynamic assemblies of cholesterol and lipids with saturated acyl chains, such as sphingolipids, glycosphingolipids, and cholesterol, in the exoplasmic leaflet of the membrane bilayer.1,2 Proteins with LR affinity include glycosyl-phosphatidylinositol–anchored proteins, doubly acylated proteins, cholesterol-linked proteins, and some transmembrane proteins.1–4 It has been shown that clustered membrane LRs form membrane platforms, which serve to recruit or aggregate various receptors, such as tumor necrosis factor (TNF) α receptors, insulin receptors, or Fas, and also aggregate various signaling molecules, such as trimeric G-proteins, sphingomyelin, tyrosine kinases, and phosphatases, resulting in activation of different signaling pathways.2–5 It has been suggested that although the dynamic clustering of lipid microdomains has been implicated in transmembrane signal transduction in many mammalian cells, little is known about the role of this LR clustering or trafficking in the regulation of endothelial function.

Numerous studies have indicated that endothelial dysfunction or injury is, importantly, involved in the development of different vascular or systemic diseases, such as atherosclerosis, hypertension, and diabetes.6,7 Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a major enzymatic source of superoxide (O2−) in vascular endothelial cells (ECs) and may contribute to the normal regulation of endothelial function, as well as endothelial injury associated with different diseases or pathological conditions.5,9 Membrane-bound NADPH oxidase catalyzes the 1-electron reduction of oxygen using NADPH as the electron donor.8,9 Similar to the situation in phagocytes, the endothelial enzyme consists of ≥5 subunits: 2 membrane-bound subunits, gp91phox and p22phox, which are transmembrane proteins that form a stable enzyme complex, namely, cytochrome b558, and 3 cytosolic subunits, p47phox, p40phox, and p67phox, which are localized in cytosol in resting cells and may be translocated to cytochrome b558 to function as an oxidase.8–11 In addition to these subunits, the GTPase Rac plays an important role in the activation of NADPH oxidase by assembling NADPH oxidase complex on the membrane.8–11

Received May 18, 2005; first decision June 6, 2005; revision accepted August 31, 2005.
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Hypertension is available at http://www.hypertensionaha.org
DOI: 10.11611161.01.HYP.0000196727.53300.62
In general, different stimuli activate NADPH oxidase by aggregating its subunits. Recent studies from our laboratory and others have demonstrated that various death receptor ligands or stimulators, such as TNF-α and endostatin, stimulate O₂⁻ production via activation of NADPH oxidase in ECs resulting in endothelial dysfunction. However, the mechanism by which NADPH oxidase activation couples to the death receptor-mediated signaling remains poorly understood. Here, we investigated whether LR clustering contributes to endothelial dysfunction induced by different death receptor ligands, because activation of different death receptors and consequent induction of cell death were previously attributed to LR clustering. We tested the hypothesis that LR clustering is one of the earliest responses of ECs to various apoptotic factors including Fas ligand (FasL), TNF-α, and endostatin, which results in NADPH oxidase aggregation and activation and consequent endothelial dysfunction. We first characterized the formation of LR clusters in coronary ECs in response to different stimuli. We then examined the role of LR clustering in aggregation and activation of NADPH oxidase in EC membrane. Furthermore, we determined whether LR clustering contributes to endothelial dysfunction by measuring endothelium-dependent vasodilation in isolated perfused small arterial preparation in the presence of LR disrupters.

Methods

Cell Culture and Reagents

Bovine coronary arterial ECs were isolated and maintained in RPMI 1640 (Invitrogen) containing 15% FBS (HyClone), 1% antibiotics (Sigma), and 1% glutamine (Invitrogen) as described.

Immunofluorescence Analysis of LR Clusters

Individual LRs are too small (~50 nm) to be resolved by standard light microscopy; however, if raft components are cross-linked in living cells, clustered raft protein and lipid components can be visualized by fluorescence microscopy. For microscopic detection of LR platforms or LR-associated proteins, ECs were grown on poly-L-lysine–coated glass coverslips and treated with the following death receptor agonists: FasL (10 ng/mL, 15 minutes, Upstate) with or without preincubation of nystatin (10 μg/mL, Sigma) and methyl-β-cyclodextrin (MCD, 1 mmol/L, Sigma) for 20 minutes, anti-Fas CH11 (a stimulatory antibody for Fas activation, 100 ng/mL, 5 minutes, Upstate), TNF-α (10 ng/mL, 15 minutes, Sigma), endostatin (1 μg/mL, 15 minutes, Calbiochem), or a cross-linking cholera toxin antibody (CTX-Ab, 1:200 dilution, 15 minutes, Molecular Probes) to induce LR clustering. ECs were then washed in cold PBS and fixed for 15 minutes in 1% paraformaldehyde in PBS and blocked with 1% BSA in cell culture medium for 30 minutes. GSH-gangliosides enriched in LRs were stained with FITC-labeled cholera toxin (CTX; 1 μg/mL, 15 minutes, Molecular Probes). ECs were washed in cold PBS, fixed in 1% paraformaldehyde for another 10 minutes, and mounted on glass slide with VECTSHIELD mounting media (Vector Laboratories). Staining was visualized using a conventional Zeiss fluorescence microscope or a Leica TCS SP2 scanning confocal microscope. The patch formation of FITC-labeled CTX and gangliosides complex represented the clusters of LRs. Clustering was defined as 1 or several intense spots of fluorescence on the cell surface, whereas unstained cells displayed a homogenous distribution of fluorescence throughout the membrane. In each experiment, the presence or absence of clustering in samples of 200 cells was scored by 2 independent observers. The results are given as the percentage of cells showing a cluster after the indicated treatment as described. In the case of dual staining, ECs were incubated for 1 hour at room temperature with 1 μg of the indicated primary monoclonal mouse antibodies followed by incubation with 5 μg/mL Texas Red–conjugated anti-mouse antibody (Santa Cruz) for an additional 2 hours at room temperature. FITC-CTX staining was then performed as described above. Negative control staining was performed with irrelevant monoclonal antibodies.

Preparation of Plasma Membrane

Cell membranes were prepared as described previously. The protein concentrations were determined by the method of Bradford (Bio-Rad protein assays). To evaluate the purity of the membrane fraction, the activity of lactate dehydrogenase, a marker enzyme for the cytosol, was assessed using a Sigma diagnostic kit.

Isolation of LR Microdomains by Gradient Centrifugation

LR microdomains were isolated as described. Cells were lysed in 1.5 mL MBS buffer containing (in micromoles per liter) morpholinoethane sulfonic acid, 25; NaCl, 150; EDTA, 1; PMSF, 1; Na3VO4, 1; and a mixture of “complete” protease inhibitors (Roche) and 1% Triton X-100 (pH 6.5). Cell extracts were homogenized by 5 passages through a 25-gauge needle. Homogenates were adjusted with OptiPrep Density Gradient medium (Sigma) to 40% and overlaid with equal volume of 4.5 mL of discontinuous 30%/5% OptiPrep Density Gradient medium. Samples were centrifuged at 32,000 rpm for 30 hours at 4°C using a SW32.1 rotor (Beckman). Ten fractions were collected from the top to the bottom (fraction numbers 1 to 10). For immunoblot analysis, proteins of each fraction were precipitated by mixing with equal volume of 30% trichloroacetic acid and 30 minutes of incubation on ice. Proteins were spun down by centrifugation at 13,000 rpm at 4°C for 15 minutes. The protein pellet was carefully washed with cold acetone twice, air dried, and then resuspended in 1 mol/L Tris-HCl (pH 8.0), which were ready for immunoblot analysis.

Western Blot Analysis

For immunodetection of LR-associated proteins, 50 μL of resuspended proteins were subjected to SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked as described. The membrane was probed with primary monoclonal antibodies anti-flotillin-1, anti-gp91phox, anti-p47phox (1:1000, BD Biosciences), or anti-Rac (1:1000, Upstate) overnight at 4°C followed by incubation with horseradish peroxidase–labeled anti-mouse IgG (1:5000). The immunoreactive bands were detected by chemiluminescence methods (Pierce) and visualized on Kodak Omat film.

NADPH Oxidase Assay

A dihydroethidium (DHE)-based fluorescence spectrometric assay was used to assess O₂⁻ production from NADPH oxidase. DHE is oxidized specifically by O₂⁻ to yield ethidium, which binds DNA and has fluorescence at excitation/emission of 480/610 nm. Briefly, 20-μg proteins of membrane fractions or 50 μL of LR fractions were incubated with 100 μmol/L DHE and 0.5 mg/mL salmon test DNA (binds ethidium to amplify fluorescence signal) in 200 μL of PBS. NADPH (1 mmol/L) was added immediately before recording ethidium fluorescence by a fluorescence microplate reader (FLX800, Bio-Tek). The ethidium fluorescence increase (arbitrary unit) was used to represent NADPH oxidase activity.

Isolated Small Coronary Artery Preparation

Fresh bovine hearts were obtained from a local abattoir. The left ventricular wall was rapidly dissected and immersed in ice-cold physiological saline solution as described previously. Small intramural coronary arteries (100 to 200 μm ID) were carefully dissected and cannulated. The arteries were pressurized to 60 mm Hg and equilibrated in physiological saline solution at 37°C for 1 hour. The internal diameter of the arteries was measured with a video recording system. All of the drugs were added into the bath solution unless otherwise indicated. The vasodilator response to bradykinin (BK, 10⁻⁹ to 10⁻⁶ mol/L) was expressed as the percent relaxation of U-46619-induced precontraction based on changes in the ID.
Data are presented as mean±SE. Significant differences between and within multiple groups were examined using ANOVA for repeated measures, followed by Duncan’s multiple-range test. Student t test was used to evaluate the significant differences between 2 groups of observations. P<0.05 was considered statistically significant.

Results
Detection of Agonist-Induced LR Clustering in EC Membrane
Figure 1A presents typical fluorescence images for LR clusters as shown by a FITC-CTX–labeled fluorescent patch on the EC membrane. Under resting conditions, we found that LRs distributed through the EC membrane as indicated by weak diffused green FITC fluorescence in a random punctuate staining pattern (control). After Fas activation, however, green fluorescence patches were detected in the EC membrane (FasL). Figure 1B summarizes the effects of different death receptor ligands or agonists on the LR clustering in EC membranes by counting these LR clusters or patches. Under resting condition, only 19.4% of the cells displayed with intense LR clusters, whereas 70.6% displayed after treatment with FasL. Disruption of LRs by nystatin or MCD, which interfere with cholesterol in rafts and, thus, destroy membrane rafts, decreased FasL-induced clustering of LR clusters by 80% and 71%, respectively. Similar to FasL, the agonistic anti-Fas antibodies CH11, CTX-Ab, TNF-α, and endostatin increased LR clusters to 69.1%, 68.9%, 58.9%, and 63.3%, respectively. Because FasL has the most potent effect to produce LR clusters on ECs, this compound was used as a prototype of activators of death receptors to perform the subsequent experiments.

Colocalization of gp91phox Within LR Clusters
To examine whether NADPH oxidase components aggregate in the LR clusters to form a redox signaling platform, we stained ECs with Texas Red–conjugated anti-gp91phox and FITC-CTX, and the distribution of gp91phox within LRs clusters was visualized in the EC membrane. Similar to FITC-CTX labeling, gp91phox evenly spread throughout the membrane under normal condition (Figure 2, control). FasL aggregated gp91phox as shown by red fluorescence patches. FITC-CTX and gp91phox colocalized as indicated after merging the fluorescence stainings. Pretreatment of ECs with MCD or nystatin to disrupt rafts abrogated patching and clustering of both FITC-CTX and gp91phox after FasL stimulation.

gp91phox, p47phox, and Rac Aggregate in Isolated LR Microdomains
As shown in Figure 3A, Western blot analysis detected positive expression of flotillin-1 in fractions 3 to 5, which were referred to lipid raft fractions previously.23 Figure 3B
shown that gp91<sub>phox</sub> could be detected in most of the membrane fractions from ECs; however, there was a marked increase in gp91<sub>phox</sub> protein in LR microdomains when ECs were stimulated by FasL, and this increase was significantly inhibited by pretreatment with nystatin. Similarly, p47<sub>phox</sub>, a cytosolic NADPH oxidase subunit, and Rac, a cytosolic small G protein, were also found increased in LR fractions on FasL stimulation, which were inhibited by nystatin (Figure 3C and 3D).

NADPH Oxidase Activity in LR Microdomains of EC

Next we analyzed the NADPH oxidase activity present in both whole membrane and LR fractions from control and FasL-treated ECs by fluorescent spectrometry of DHE oxidation and DNA binding. We found that FasL significantly increased membrane NADPH oxidase activity by 40% (Figure 4A) and by 178% in LR fractions (Figure 4B), which were both prevented by MCD pretreatment. To examine whether MCD can directly interfere with NADPH oxidase, we incubated EC homogenate with MCD, determined the NADPH oxidase activity, and found that the presence of MCD did not alter the oxidase activity in EC homogenate (data not shown). Nystatin was found to have strong autofluorescence that interfered with DHE fluorescence and, therefore, was not appropriate for this assay.

Effects of a Blockade of LR Clustering on BK-Induced Vasodilator Responses in Small Bovine Coronary Arteries

Concentration–response curves of the endothelium-dependent vasodilation induced by BK were determined in small coronary arteries before and after FasL treatment. Figure 5 shows that BK produced a concentration-dependent vasorelaxation in small coronary arteries with a maximal response at
2.3.82 ± 0.9%. Incubation of the arteries with FasL (10 ng/mL perfused into the lumen) had no significant effect on the basal arterial diameters but markedly attenuated the BK-induced increase of arterial diameters. Preincubation of the arteries with the NADPH oxidase inhibitor apocynin (100 μmol/L) in the lumen for 15 minutes reversed the inhibitory effects of FasL on the BK-induced vasorelaxation. Similarly, the inhibitory effect of FasL was restored by 10-minute preincubation of nystatin (10 μg/mL) or MCD (1 mmol/L) in the lumen. Treatment of nystatin or MCD alone had no significant effect on basal arterial diameters or BK-induced changes at the doses used in the present study.

Discussion

The present study demonstrates that various death receptor agonists induced LR clustering in coronary arterial ECs, as shown by the formation of larger membrane LR patches. FasL induced an aggregation of gp91phox in and translocation of cytosolic p47phox and Rac into LR clusters, which resulted in a dramatic increase of NADPH oxidase activity within LR microdomains. Disruption of LRs or inhibition of NADPH oxidase significantly restored the vasodilator response of small coronary arteries to BK in the presence of FasL, which impairs endothelial function.

Using confocal microscopic analysis with FITC-CTX as a marker, we first visualized LR clusters on the cell membrane of ECs. CTX relatively specifically binds to ganglioside GM1, which partitions into LRs.23,24 LRs distribute throughout the EC membrane in a random punctuate staining pattern, whereas stimulation with FasL, TNF-α, or endostatin results in the formation of multiple “nonpolarized,” CTX-positive patches. Our findings are consistent with a recent study demonstrating that LRs were stained in human umbilical vein ECs.25 Considering that the clustering of cell membrane LRs exerts important signaling action in many cell types,1–5 we suggest that LR clustering may serve as a common mechanism for transmembrane signaling, which mediates the actions of these agonists.

One of the important findings in the present study is that LR clustering is coupled to the NADPH oxidase aggregation on the EC membrane. By double staining coronary ECs with FITC-CTX and anti-gp91phox antibody, we detected gp91phox colocalized with LR elements on the cell membrane, which were clustered by FasL. Furthermore, we determined whether LRs are coupled to the aggregation of NADPH oxidase subunits. We found a significant amount of gp91phox protein in isolated LR fractions, which was additionally increased by FasL. Two important regulators, cytosolic subunit p47phox and a small G protein, Rac, translocated into membrane rafts on Fas stimulation, which is consistent with a recent report that the association of p47phox with gp91phox occurs in LRs in neutrophils.23 Although gp91phox and p47phox were detected in fractions other than LRs under resting condition, the marked increases in gp91phox and p47phox proteins in isolated LR fractions by FasL reflect a shift of dynamic balance of these NADPH oxidase subunits between rafts and other regular fractions when ECs are challenged with various stimuli. Because many stimuli activate NADPH oxidase by aggregating its subunits, and translocation of p47phox and Rac to the membranes is closely associated with this aggregation and consequent activation,12 the increases in p47phox and Rac in the LRs fraction may indicate the activation of this oxidase. Taken together, the results from the present study confirm an important coupling of LRs with NADPH oxidase subunits in coronary ECs.

There is a concern about the subtype of LRs involved in the aggregation and activation of NADPH oxidase. Generally, there are 2 types of LRs: caveolar and noncaveolar rafts. Caveolae are a subset of plasma membrane rafts in cell surface invaginations. They exist in cell types that express caveolins, which polymerize to bend the membrane to form caveolae.26 However, the lipid components in caveolae or noncaveolar rafts are difficult to differentiate, and the concept of caveolae as a subset of LR is still controversial.1,26 Therefore, the present study did not intend to investigate which subtype is involved. Recent studies have indicated that the formation of caveolae may be associated with nitric oxide production and endocytosis in ECs.27,28 It is possible that caveolae and noncaveolar LRs and their clustering mediate different signaling pathways, whereby participating in the regulation of different cell functions or cell responses in the same type of cells. Because apoptotic factors, such as TNF-α, have been shown to alter endothelial function independent of the caveolae-related mechanism, and noncaveolar LR clustering has been implicated in mediating the actions of these factors in other cell types, it is likely that noncaveolar LR clustering may play a role in the aggregation of membrane NADPH oxidase components in ECs.5

To provide direct evidence that aggregation of NADPH oxidase components via LR clustering is involved in the activation of this enzyme, we isolated the whole membrane fractions, as well as its LR fractions, from ECs and determined NADPH oxidase-derived O2− production. FasL enhanced NADPH oxidase activity in the whole membrane fraction, which was prevented by MCD. In LR fractions, FasL more dramatically increased NADPH oxidase activity, which was blocked by LR disruption with MCD. This is similar to the results obtained from other cells, such as T cells.29 These results support the view that LR clustering contributes to the aggregation and activation of NADPH oxidase in ECs when they were exposed to FasL or other stimuli.

With respect to the physiological significance of this LR clustering–mediated NADPH oxidase activation, it is very likely that this signaling pathway may importantly contribute to cell apoptosis of coronary ECs. The death factors, including FasL, TNF-α, and endostatin, all stimulate cell apoptosis and participate in the development of different pathological injuries, such as ischemia and reperfusion, atherosclerosis, arterial inflammation, and thrombosis.15–17,30 However, in in vitro experiments, these factors induced detectable apoptosis of ECs after 6 to 10 hours of incubation.15–17,30 It has been shown that these apoptotic factors can rapidly alter the endothelial function before apoptosis, and endothelial dysfunction is considered an early effect of these factors.13,14 Therefore, we were wondering whether LR clustering–mediated activation of NADPH oxidase is responsible for endothelial dysfunction induced by FasL or other death factors.
Using freshly isolated perfused small coronary arteries, we found that FasL, added in the arterial lumen impaired the endothelium-dependent vasodilator response to BK, which was similar to our previous results obtained with TNF-α. This effect of FasL on BK-induced, endothelium-dependent vasodilation was recovered by NADPH oxidase inhibition using apocynin, suggesting that NADPH oxidase-derived O₂⁻ is involved. Similarly, when the arteries were pretreated by the disruption of LRs using nystatin and MCD, FasL-induced impairment of endothelium-dependent vasodilation was also significantly attenuated. Taken together, these results confirm that LR clustering coupled aggregation, and activation of NADPH oxidase may be a contributing mechanism for FasL-mediated endothelial injury and dysfunction. It should be noted that the disruption of LR clusters by nystatin, an antifungal agent, which can perforate the cell membrane, causes ion exchange, such as Ca²⁺ influx or K⁺ efflux, and thereby affects vascular tone. In addition, nystatin has been reported to dissociate caveolins from plasma membrane lipid-rich domains, in which endothelial nitric oxide synthase locates. This may result in the activation of endothelial nitric oxide synthase and bypass LR signaling to enhance BK-induced vasodilation. However, in the present study, we did not find that nystatin produces any significant changes on basal vascular tone, nitric oxide production, and vasodilator response to BK at the doses used. Therefore, the recovery of vasodilator response to BK in FasL-treated small coronary arteries by nystatin seems to be associated with its LR disruption and consequent inhibition of NADPH oxidase activity, which was, indeed, confirmed in our isolated LR experiments.

The present study did not attempt to explore the mechanism by which LRs are clustered in coronary ECs. Recent studies have demonstrated that LR clustering is associated with production or action of ceramide, a sphingomyelin metabolite, around cell membrane. It has been reported that ceramide is produced in response to different injury factors, especially those apoptotic factors including FasL, TNF-α, and endostatin, and acts as a signaling molecule to activate LR clustering. Ceramide leads to activation of NADPH oxidase, resulting in endothelial dysfunction in small coronary arteries. It is possible that ceramidated-induced LR clustering is an important mechanism mediating its action to activate NADPH oxidase. Taken together, it can be proposed that various death receptor agonists stimulate ceramide production, which mediates fusion of LRs to membrane platforms and aggregation of NADPH oxidase and thereby stimulates the production of O₂⁻, resulting in endothelial dysfunction.

In summary, the present study demonstrated that LRs cluster in coronary ECs in response to various death receptor agonists. This membrane molecular trafficking mechanism results in NADPH oxidase subunit aggregation and activation of this enzyme and forms a redox signaling platform on the membrane of ECs, increasing O₂⁻ production and impairing endothelial function.

**Perspectives**

The results from the present study might increase our understanding of the signaling mechanism mediating the early response of the death receptors in ECs and may provide a new insight into the regulation of endothelial function. It is plausible that the formation of this redox signaling platform importantly contributes to the normal regulation of endothelial function and, in particular, to endothelial dysfunction associated with various death receptor agonists. Given that these death receptor agonists, such as TNF-α or FasL, are importantly involved in the pathogenesis of different vascular diseases, such as atherosclerosis, hypertension, and ischemia/reperfusion injury, the formation of this redox signaling platform on the membrane of ECs may be implicated in the development of these diseases.

**Acknowledgements**

This study was supported by grants from the National Institutes of Health (HL-57244, HL-70726, and HL-51055) and American Heart Association Established Investigator Grant 9940167N (to P.L.L.) and Predoctoral Fellowship 0410061Z (to A.Y.Z.).

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_Hypertension_. 2006;47:74-80
doi: 10.1161/01.HYP.0000196727.53300.62

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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