Sphingosine Kinase Activation Mediates Ischemic Preconditioning in Murine Heart

Zhu-Qiu Jin, PhD; Edward J. Goetzl, MD; Joel S. Karliner, MD

Background—Phosphorylation of sphingosine by sphingosine kinase (SK) is the rate-limiting step in the cellular synthesis of sphingosine 1-phosphate (S1P). The monoganglioside GM1, which stimulates SK, is cardioprotective in part through increased generation of S1P that protects myocytes by diverse mechanisms. Because protein kinase C (PKCε) activation is necessary for myocardial ischemic preconditioning (IPC) and PKC activators increase SK activity, we tested the hypothesis that SK may be a central mediator of IPC.

Methods and Results—In adult murine hearts, IPC sufficient to reduce infarct size significantly increased cardiac SK activity, induced translocation of SK protein from the cytosol to membranes, and enhanced cardiac myocyte survival. IPC did not increase SK activity in PKCε-null mice. The SK antagonist N,N-dimethylsphingosine inhibited PKCε activation and directly abolished the protective effects of IPC and the enhanced SK activity induced by IPC.

Conclusions—These findings demonstrate that PKCε is thus recruited by IPC and induces activation of SK that then mediates IPC-induced cardioprotection in murine heart. (Circulation. 2004;110:1980-1989.)

Key Words: ischemia • myocardial infarction • enzymes • signal transduction

Ischemic preconditioning (IPC) is defined as a brief period of myocardial ischemia/reperfusion (IR) that significantly reduces injury resulting from subsequent longer-term IR. This phenomenon has been observed in different animal species (dog, rabbit, pig, rat, and mouse) and in patients. Both triggers and mediators have been identified for IPC. Specific triggers include adenosine via A1 and A2 receptors, bradykinin via the B2 receptor, opioids via the δ-receptor) are recognized as triggers of IPC. Among molecules implicated in mediating IPC are protein kinase Cε (PKCε), p38 mitogen-activated protein kinase, extracellular signal–regulated kinases (ERK)1/2, phosphatidylinositol 3-kinase (PI3K), and protein tyrosine kinases. Sphingosine-1-phosphate (S1P) is a lipid-signaling molecule involved in the regulation of cellular growth, viability, mitosis, and angiogenesis. S1P acts either by liganding with G protein–coupled receptors or intracellularly as a second messenger. S1P receptors transduce cellular signals in part through PKCε, ERK 1/2, and PI3K. S1P also plays an important role in cardiovascular development. Previously we showed that exogenously administered S1P protects the heart against IR injury, an effect that is independent of PKCε. In contrast, the naturally occurring ganglioside GM1, a sphingosine kinase (SK) activator, exerted cardioprotection through a PKCε-dependent mechanism. SK generates S1P by phosphorylation of sphingosine. There are 2 different isoforms of SK (SK1 and SK2). Although both are expressed in adult mouse heart, SK1 appears to be the major isoform. Transfected cell lines in which SK was overexpressed exhibited increased intracellular S1P content associated with enhanced cell growth and survival. Recently, it has been shown that PKCε activation significantly increased SK activity and caused SK protein translocation from the cytosol to plasma membranes. Moreover, PKCε is a well-recognized mediator of IPC, but PKCε targets other than mitochondrial KATP channels have eluded detection. On the basis of these considerations, we have used an ex vivo mouse model to test the hypothesis that PKCε-induced SK activation is involved in mediating cardiac IPC.

Methods

Animal Procedures

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, Washington DC, 1996), and the Animal Care Subcommittee of the San Francisco Department of Veterans Affairs Medical Center approved all procedures.

SK Activity Assay

Isolated mouse hearts were mounted on a modified Langendorff apparatus as previously described from our laboratory. Hearts were subjected to IPC (see later section) consisting of 2 minutes of global ischemia and 5 minutes of reperfusion. N,N-Dimethylphosphinosine (DMS, Biomol), 1 μmol/L or 10 μmol/L, or ganglioside GM1 (Sigma-Aldrich), 10 nmol/L, was infused for 5 or 2 minutes, respectively. After treatments, all hearts were placed in liquid N2 and stored at −80°C until SK assay. Hearts were thawed and homoge-
nized in assay buffer (200 μL of 1 mol/L Tris-HCl, pH 7.4; 100 μL of 0.1 mol/L EDTA; 50 μL of 100 mmol/L deoxypyridoxine; 150 μL of 1 mol/L NaF; 0.7 μL of 2-mercaptoethanol; 50 μL of 0.2 mol/L sodium orthovanadate; 10 μL of 10 mg/mL leupeptin and aprotinin; 10 μL of 10 mg/mL trypsin inhibitor; 86.4 mg β-glycerophosphate; 20 μL of 0.2 mol/L phenylmethylsulfonyl fluoride; 4 mL of 50% glycerol; and 5.36 mL water as described). The homogenate was centrifuged at 100,000g for 20 minutes, and the supernatant was collected and used for SK assay.

The reaction mixture (200 μL) for the SK assay contained 16.7 μmol/L [3H]sphingosine (0.2 μCi, Perkin-Elmer), 80 mmol/L Tris-HCl, 20 mmol/L MgCl₂, 1 mmol/L ATP, 4 mg/mL bovine serum albumin, and 200 μg heart tissue protein. The mixture was incubated for 1 hour at 37°C. The Eppendorf tube containing the reaction mixture was then placed on ice, and 20 μL of 1 mol/L HCl was added to stop the reaction. The [3H]S1P formed was extracted by chloroform/methanol/HCl (100:200:1, vol/vol/vol). The resultant lower chloroform-phase samples were analyzed for [3H]S1P formation from [3H]sphingosine by thin-layer chromatography with the use of thin-layer chromatography plates coated with silica gel (Whatman Ltd), developed in butanol/acetic acid/water (3:1:1, vol/vol/vol), and visualized with ninhydrin (Sigma-Aldrich). Sphingosine (Rf value, 0.58±0.015; Sigma-Aldrich) and S1P (Rf value, 0.31±0.012; Biomol) were used as reference standards. [3H]enhancer (Perkin-Elmer) was applied to increase the autoradiography intensity. The spots were scraped off, and radioactivity was measured by liquid scintillation counting (LS 6500 multipurpose scintillation counter, Beckman).

**PKCe-Null Mice**

PKCe-null mice were obtained from Dr Robert Messing (Gallo Research Center, Emeryville, Calif) as previously described from our

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**Figure 1.** Effect of IPC on SK activation in supernatant (cytosolic) fraction of mouse heart. A, Representative autoradiographic image of [3H]sphingosine and [3H]S1P. NC indicates normal control. B, Scintillation counting results of [3H]sphingosine, [3H]S1P, and [3H]sphingosine/ [3H]S1P formation in control hearts and hearts subjected to IPC. Abbreviations are as defined in text. Data are expressed as mean±SEM, n=4. *P<0.05 vs control group.
laboratory. These mice along with their wild-type littermates were used for studies of SK activity and SK protein expression in both cytosolic and membrane fractions. Only male mice were used for all studies. Genotyping by polymerase chain reaction to confirm the absence of PKC\(\varepsilon\) DNA was routinely performed on tail samples.

**Western Blot Analysis**

Measurement of SK protein was performed by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis Western blot techniques. In brief, 50 \(\mu\)g of protein derived from either the cytosolic or the membrane fraction of each homogenate was electrophoresed on a 10.0% denaturing gel at 30 mA per lane for 2 hours. Proteins were electrotransferred onto a nitrocellulose membrane (Bio-Rad) at 200 mA for 1.5 hours. The transfer efficiency was checked by Ponceau S (Sigma-Aldrich). Adequate background blocking was accomplished by incubating the nitrocellulose membrane with 5% nonfat dry milk in phosphate buffer solution (pH 7.4). Goat polyclonal antibody to SK1 (Novus Biologicals, Inc) was used to measure the expression of SK1. Rabbit polyclonal antibody against phospho-PKC\(\varepsilon\) (Ser 729, Upstate Biotechnology) was used to detect the expression of this PKC isoform in the membrane fraction. The immunoreactive bands were quantified by densitometric analysis of digitized autoradiograms with NIH Image 1.61 software.

**Langendorff Isolated, Perfused Heart Preparations**

Male C57BL/6J mice (4 months of age, weighing 28 to 30 g) were heparinized (500 U/kg IP) and anesthetized with sodium pentobarbital (60 mg/kg IP). Hearts were rapidly excised, washed in ice-cold arresting solution (NaCl 120 mmol/L, KCl 30 mmol/L), and cannulated via the aorta on a 20-gauge stainless steel blunt needle. Hearts were perfused at 70 mm Hg on a modified Langendorff apparatus with Krebs-Henseleit solution containing (mmol/L) NaCl 118, KCl 4.7, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 24, glucose 5.5, sodium pyruvate 5.0, and EDTA 0.5 bubbled with 95% O\(_2\)/5% CO\(_2\) at 37°C. Platinum electrodes connected to a stimulus generator (Grass Instruments) were used to pace the hearts at 360 beats/min.

**IR and IPC Protocols**

For IR experiments, the protocol consisted of 20 minutes of global ischemia and 30 minutes of reperfusion. For IPC, hearts from 3-month-old C57BL/6J mice (Charles River Laboratories, Wilmington, Mass) and PKC\(\varepsilon\) null mice were subjected to 2 minutes of global ischemia and 5 minutes of reperfusion followed immediately by IR.

**Figure 2.** Effect of DMS on SK activity in supernatant (cytosolic) fraction of mouse heart. A, Representative autoradiographic image of [\(^{3}\)H]sphingosine and [\(^{3}\)H]S1P. B, [\(^{3}\)H]S1P/[\(^{3}\)H]sphingosine ratio and [\(^{3}\)H]S1P formation were attenuated in DMS (10 \(\mu\)mol/L for 5 minutes)-pretreated mouse heart. \(n=3\). *\(p<0.05\) vs control group. P indicates positive control. All other abbreviations are as defined in text.

**Figure 3.** Pretreatment with 10 \(\mu\)mol/L DMS for 5 minutes totally inhibited SK activity induced by IPC in both cytosolic and membrane preparations from mouse heart. *\(p<0.05\) vs control group; #\(p<0.05\) vs IPC group. \(n=4\) for each condition. Abbreviations are as defined in text.
In some experiments, hearts were pretreated with 1 μmol/L DMS (Biomol) perfusion for 10 minutes before 2 minutes of ischemia and 5 minutes of reperfusion or with 10 μmol/L DMS perfusion for 5 minutes either alone or before IPC. Hemodynamics (left ventricular [LV] developed pressure, LV end-diastolic pressure, LV \( \frac{dP}{dt}_{\text{max}} \), and coronary flow) were recorded as previously described.

**Infarct Size Determination**

After 20 minutes of global ischemia and 30 minutes of reperfusion, a subset of hearts in each group was infused with 15 mL of 1% triphenyltetrazolium chloride (Sigma) in phosphate-buffed saline at a rate of 1.5 mL/min as previously described. Hearts were then removed from the cannula, weighed, and fixed overnight in 10% formalin. Hearts were removed from formalin and stored frozen at \(-20^\circ\text{C}\) until sectioning for analysis of LV infarct size. Hearts were sliced into 2-mm transverse section from apex to base and digitally photographed on each side (Camedia E-10, Olympus Camera). Computerized area analysis was performed with NIH Image software. The infarct size of each section was expressed as a fraction of the area at risk, defined as the total area of the LV in this global ischemia model.

**Statistical Analysis**

The data are presented as mean±SEM. The significance of differences in mean values for infarct size and SK1 protein expression between groups was evaluated by 1-way ANOVA, followed by post hoc testing (Newman-Keuls test). SK activity between 2 groups was evaluated by the Student \( t \) test. Any differences between hemodynamic measurements at different time points were assessed by 2-way ANOVA with repeated measures, followed by post hoc testing for individual differences. \( p<0.05 \) was considered statistically significant.

**Results**

**IPC Enhanced SK Activity**

In initial studies, we tested the hypothesis that IPC is related to SK activation. Hearts were perfused in the Langendorff mode and subjected to IPC by a standard procedure as described in Methods. We then measured SK activity in supernatants prepared from whole-heart homogenates by thin-layer chromatography and autoradiography as described in Methods. As shown in Figure 1, IPC increased absolute levels of S1P and reduced sphingosine levels. The ratio of S1P to sphingosine was enhanced by IPC, whereas there was no difference in the total content of S1P plus sphingosine compared with control.

**DMS Reduced SK Activity**

As shown in Figure 2, we next tested the effect of the SK inhibitor DMS on SK activity. Isolated murine heart perfusion in the basal state with DMS significantly decreased cytosolic SK activity, as evidenced by a marked reduction in both the absolute amount of S1P formation and in the ratio of S1P to sphingosine. As shown in Figure 3A and 3B, DMS pretreatment abolished IPC-evoked SK activity in both cytosolic and membrane fractions. There was no difference in SK activity between the DMS+IPC group and the DMS-alone group.

**PKCε Was Necessary for IPC-Induced SK Activation but Not Protein Translocation**

Because PKC is a well-recognized activator of SK, we next asked whether PKCε is required for SK activation induced by...
IPCs. For these experiments, we prepared cytosolic and membrane fractions from wild-type littermate and PKCε-null mouse hearts. As shown in Figure 3A and 3B, IPC increased SK activity in both fractions of wild-type littermate mouse hearts. In contrast, the ability of IPC to induce SK activation was abrogated in both cytosolic and membrane fractions of PKCε-null mouse hearts (Figure 4A and 4B). We also found that SK activity at baseline was greater by 40% in the membrane fraction of the PKCε-null hearts (data not shown) but did not increase further after IPC (Figure 4B).

Others have reported translocation and activation of SK in response to PKC activation by a phorbol ester.14 To determine whether SK1 protein is translocated by IPC, we used a commercially available antibody to SK1 (Novus Biologicals, Inc). We found that IPC was indeed associated with SK1 translocation from the cytosol to the membrane fraction in both wild-type and PKCε-null mouse hearts (Figure 5). SK in the membrane fraction was increased from 15.1±1.3 to 24.9±2.2 arbitrary densitometry units (n=4, P<0.05). PKCε-null mice exhibited a virtually identical increase. Despite this similarity in SK protein translocation induced by IPC, only wild-type littermate cytosolic and membrane preparations exhibited an increase in SK activity in response to IPC (Figures 3 and 4). These observations suggest that the increase in SK activity was not due to protein translocation but rather resulted from a PKCε-mediated increase in the intrinsic catalytic activity of SK.

**IPC Activation of PKCε Was Inhibited by DMS**

Because DMS abolished the beneficial effect of IPC on infarct size and LV hemodynamics (see following section), we next wanted to learn whether this finding involved only SK activation or was the consequence of PKCε inhibition that resulted in suppression of SK activity. It has been suggested that DMS is a potent and specific inhibitor of PKC,19,20 but this effect is concentration and cell line dependent. Thus, others have reported that DMS at 1 μmol/L did not inhibit PKC in PC12 cells or Swiss 3T3 cells,21 but the IPC response to DMS has not been tested either in cardiac tissue or on activation of PKC isozymes.

Because the activity and intracellular localization of PKCε are controlled by phosphorylation at 3 highly conserved sites in the catalytic kinase domain and PKCε plays a necessary role in IPC,15,16 we measured PKCε phosphorylation in the membrane fraction of mouse hearts. As shown in Figure 6A, IPC increased phosphorylation of PKCε (Ser 729) (P<0.05, compared with the control group), and DMS pretreatment abolished this effect. No changes were noted in the cytosolic fraction (data not shown). Consistent with prior reports in the isolated rat heart,22,23 PKCε as detected by immunoblotting was not translocated to particulate fractions after IPC in our mouse heart preparations (Figure 6B). DMS suppression of IPC-induced SK activity thus may be attributable both to reduced elicitation of PKCε, which is responsible for SK activation, and to direct inhibition of the SK catalytic function. The concurrent effects of DMS on IPC-evoked phosphorylation of PKCε and on IPC augmentation of SK activity provide further evidence for an early role of PKCε in the entire sequence of biochemical events, but this requires more rigorous proof. One approach would be to use PKCε-null mice, but to our knowledge, such a model is not currently available for study.

**Cardioprotection Induced by IPC Was Abolished by DMS**

As shown in Figure 7, infarct size was reduced by IPC from 45.1±4.5% in the IR group to 14.6±0.9% of the risk area in the IPC group (n=5, P<0.05 versus IR). After perfusion with 1 μmol/L DMS, the infarct size reduction induced by IPC was abolished (49.7±3.8% in the DMS+IPC group, P<0.05 versus the IPC-only group). As we previously reported,9 both 10 nmol/L SIP and 10 nmol/L GM, pretreatment decreased infarct size to levels similar to those induced by IPC, and additional separate experiments confirmed these observations (data not shown). Both we and others have previously...
noted\(^9\)–\(^24\) that IPC consisting of 2 minutes of ischemia and 5 minutes of reperfusion not only significantly reduced infarct size but also prevented LV dysfunction induced by 20 minutes of global ischemia and 30 minutes of reperfusion. In separate experiments, data obtained in this study were consistent with these reports (Table).

**Discussion**

The major new finding of this study identifies SK activation in myocardial IPC. Our data further indicate that PKCe is necessary for the activation of SK induced by IPC and thereby implicates SK as a heretofore-unrecognized target of PKCe in IPC. These observations are based on the following evidence: (1) IPC significantly increased cardiac SK activity and enhanced myocardial survival. (2) Increased SK activity induced by IPC was abolished in PKCe-knockout mice. (3) DMS inhibited both SK and PKCe activation and abolished the cardiac protection induced by IPC. (4) Our previous data\(^9\) showed that the monosialoganglioside GM\(_1\), which activates SK in the heart,\(^25\) produced cardioprotection, and S1P, the metabolic product of SK activation, mimics the beneficial effect on the heart induced by IPC.

**Regulation of SK**

Two isoforms of mammalian SK (SK1 and SK2) have been cloned and characterized.\(^10\)–\(^11\) Both SK1 and SK2 have been...
identified in adult mouse heart, where SK1 is the predominant isoform. Generally considered to be a cytosolic protein that is constitutively active, SK is thought to be largely responsible for generating basal levels of S1P within the cell. It is also thought to be a regulated protein in which incubation of cells with SK activators results in a transient increase in S1P content over basal levels, thereby initiating a cell-signaling cascade leading to diverse biological processes, including calcium mobilization, cell growth, cell survival, and angiogenesis.

Several G protein–coupled receptor agonists have been reported to activate SK, including acetylcholine, bradykinin/B2 receptor, lysophosphatidic acid, and S1P itself. Activation of PKC has been reported to enhance SK activity and result in augmented levels of S1P. Because S1P exhibits cardioprotective properties, we set out to determine whether SK is involved in IPC and whether inhibition of SK could affect IR after IPC.

PKC, SK, and IPC

PKC activation is a key component in the pathway of IPC. PKC activators mimic preconditioning, whereas PKC inhibitors block the beneficial effect of IPC. One PKC isoform, PKCε, has received particular attention as a mediator of IPC. IPC increased PKCε translocation from the cytosol to membranes. A selective PKCε antagonist, εV1-2 peptide, inhibited translocation of PKCε and abolished the protection of hypoxic preconditioning in cardiac myocytes. Although intracellular targets of PKC activation have not been precisely identified, much work has focused on mitochondrial KATP channels.

Our present study shows that IPC strongly activated SK, increased S1P levels, and decreased sphingosine content in the murine heart (Figure 1). To determine the mechanism of this activation, we focused on PKC, particularly the PKCε isoform, on the basis of its properties as a mediator of IPC. As noted earlier, PKC activation can enhance SK activity and increase S1P formation in a variety of cells. Thus, Cavallini et al showed that the monosialoganglioside GM1 protected isolated rat heart fibroblasts from apoptosis and activated SK. Ganglioside GM1 also increased SK activity in Swiss 3T3 fibroblasts. We previously showed that pretreatment with ganglioside GM1 protected isolated mouse hearts against IR, an effect that was abolished in the PKCε-null mouse. Recently, in HEK293 cells, Johnson et al showed
that phorbol myristate acetate, a direct PKC activator, stimulates SK activity accompanied by translocation of SK to the plasma membrane and increased S1P secretion into the media. This observation provides further evidence that SK is a direct substrate of PKC.

PKC, SK, and DMS

DMS, the N-methylated product of sphingosine, is a potent SK inhibitor in cell or cell-free systems. For example, the Ki of DMS for SK in cytosolic extracts of selected cell lines ranges between 2.3 and 3.1 μmol/L. However, DMS in 1 study, even at a concentration of 10 μmol/L, had no effect on PKC activity and membrane translocation. We previously reported that in neonatal rat ventricular myocytes, 10 μmol/L DMS induced cell death that could be prevented by concurrent incubation with either S1P or GM1. Thus, DMS at relatively high concentrations has been thought to act as a specific competitive inhibitor of SK in diverse cell types and has been considered to be a useful tool to elucidate the role of S1P as an intracellular second messenger. These studies did not exclude the possibility that DMS was also inhibitory to PKCe, which has been shown to be upstream of mitochondrial KATP channel activation in IPC. In this connection, others have suggested that DMS is a potent and specific inhibitor of PKC in vitro. Confirming the importance of PKCe in IPC, Gray et al recently reported that PKCe-null mouse hearts do not respond to IPC before IR. We now find that the mechanism whereby IPC activates SK is dependent

Cardiac Functional Parameters at Baseline and the End of IR Injury

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<th>Group</th>
<th>n</th>
<th>LVEDP, mm Hg</th>
<th>LVDP, mm Hg</th>
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<th>−dP/dtmax, mm Hg/s 10^3</th>
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EDP indicates end-diastolic pressure; DP, developed pressure; and CF, coronary flow. All other abbreviations are as defined in text.

*D<0.05 compared with control group.

 IPC

Figure 8. Proposed signaling cascade depicting role of SK in IPC. Role of SK as a target of PKCe is emphasized. In this pathway, IPC acts to release ligands such as adenosine, bradykinin, and opioids that bind to their G protein–coupled receptors labeled A1, B2, and δ-opioid R, respectively. This results in synthesis of diacylglycerol and PKCe activation. PKCe can either activate SK intracellularly or alter membrane translocation of both molecules, resulting in S1P synthesis. S1P either can act intracellularly by undefined mechanisms or can be exported from cell, where it binds specific G protein–coupled receptors on the cell surface, resulting in a signaling cascade that favors cell survival. Not shown are additional or alternative pathways for PKCe that involve mitochondrial KATP channel opening. GPCR indicates G protein–coupled receptor; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol-1,4,5-trisphosphate; and PLC, phospholipase C. All other abbreviations are as defined in text.
on PKCe, as demonstrated by the absence of SK activation in the PKCe-null mouse heart (Figure 4). Thus, our data indicate that at least in murine heart subjected to IPC, DMS inhibits SK by blocking the activation of PKCe.

Conclusions

On the basis of the results of this study, a proposed signaling pathway is shown in Figure 8. Short IR cycles produce adenosine, bradykinin, or opioid receptor agonists (receptor-dependent triggers) and reactive oxygen species or nitric oxide (receptor-independent triggers). These substances activate PKCe and lead to PKCe translocation from the cytosol to membrane. Activated PKCe increases SK activity in both the cytosol and membrane fractions and results in enhanced S1P formation. S1P, the metabolic product of SK, is an important second messenger that directly acts on G protein–coupled S1P receptors and/or functions as an intracellular second messenger. Enhanced S1P formation measured by high-performance liquid chromatography has also been demonstrated in biopsies of isolated rat heart subjected to IPC and IR preceded by IPC, but subcellular fractionation of S1P and SK measurements were not reported. Although the precise pathway by which S1P influences survival in cardiac (or other) cells has not been elucidated, S1P has clearly been shown by us and others to enhance cardiac survival in response to IR. The beneficial effect of S1P on cardiac myocyte survival is dependent on K(A) channel activation. The present study identifies for the first time the key role played by SK in the IPC-mediated survival pathway and implicates SK as an alternative or additional target for PKCe as part of the cardioprotective mechanism of IPC.

Acknowledgment

This work was supported by Program Project Grant 1P01HL068738-01A1 (to J.S.K.) from the National Heart, Lung, and Blood Institute.

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Circulation. 2004;110:1980-1989; originally published online September 27, 2004;
doi: 10.1161/01.CIR.0000143632.06471.93
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

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