Selective Pharmacological Agents Implicate Mitochondrial but Not Sarcolemmal K\text{ATP} Channels in Ischemic Cardioprotection

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**Background**—Pharmacological evidence has implicated ATP-sensitive K\text{+} (K\text{ATP}) channels as the effectors of cardioprotection, but the relative roles of mitochondrial (mitoK\text{ATP}) and sarcolemmal (surfaceK\text{ATP}) channels remain controversial.

**Methods and Results**—We examined the effects of the K\text{ATP} channel blocker HMR1098 and the K\text{ATP} channel opener P-1075 on surfaceK\text{ATP} and mitoK\text{ATP} channels in rabbit ventricular myocytes. HMR1098 (30 \text{mM}) inhibited the surfaceK\text{ATP} current activated by metabolic inhibition, whereas the drug did not blunt diazoxide (100 \text{mM})-induced flavoprotein oxidation, an index of mitoK\text{ATP} channel activity. P-1075 (30 \text{mM}) did not increase flavoprotein oxidation but did elicit a robust surfaceK\text{ATP} current that was completely inhibited by HMR1098. These results indicate that HMR1098 selectively inhibits surfaceK\text{ATP} channels, whereas P-1075 selectively activates surface K\text{ATP} channels. In a cellular model of simulated ischemia, the mitoK\text{ATP} channel opener diazoxide (100 \text{mM}), but not P-1075, blunted cellular injury. The cardioprotection afforded by diazoxide or by preconditioning was prevented by the mitoK\text{ATP} channel blocker 5-hydroxydecanoate (500 \text{mM}) but not by the surfaceK\text{ATP} channel blocker HMR1098 (30 \text{mM}).

**Conclusions**—The cellular effects of mitochondria- or surface-selective agents provide further support for the emerging consensus that mitoK\text{ATP} channels rather than surfaceK\text{ATP} channels are the likely effectors of cardioprotection.

**(Circulation. 2000;101:2418-2423.)**

**Key Words:** mitochondria ■ potassium ■ ischemia ■ preconditioning

Brief ischemic episodes protect the heart from subsequent lethal ischemic injury (ischemic preconditioning, IPC).\(^1\) Although the precise mechanism of IPC remains elusive, much attention has focused on the potential role of ATP-sensitive K\text{+} (K\text{ATP}) channels as the effectors of protection. Cardiac myocytes contain 2 distinct K\text{ATP} channels: the classic one in the surface membrane (surfaceK\text{ATP} channel);\(^2\) and another in the mitochondrial inner membrane (mitoK\text{ATP} channel).\(^3\) Although the cardioprotective effects were initially attributed to surfaceK\text{ATP} channels, the effects of surfaceK\text{ATP} channels on excitability cannot account for the protection.\(^4\)–\(^7\) Recent studies provide further evidence that mitoK\text{ATP} channels rather than surfaceK\text{ATP} channels are the dominant players. Diazoxide, a selective mitoK\text{ATP} channel opener in cardiac myocytes, is cardioprotective.\(^8\)–\(^9\) The mitoK\text{ATP} channel blocker sodium 5-hydroxydecanoate (5HD)\(^10\) can prevent diazoxide-induced cardioprotection\(^8\) and can block genuine IPC.\(^12\)–\(^14\) Activation of protein kinase C, which figures prominently in the signal transduction cascade of IPC, potentiates mitoK\text{ATP} channel opening.\(^10\) In light of such new information, the role of surfaceK\text{ATP} channels in cardioprotection needs to be reevaluated.

A selective opener and a blocker of mitoK\text{ATP} channels, namely diazoxide and 5HD, have been identified. A missing link, however, has been the absence of selective agonists or antagonists of surfaceK\text{ATP} channels. In the present study, we first examined the effects of the K\text{ATP} channel blocker HMR1098\(^15\) and the K\text{ATP} channel opener P-1075\(^16\) on K\text{ATP} channels in rabbit ventricular myocytes. Our results show that both HMR1098 and P-1075 target surfaceK\text{ATP} but not mitoK\text{ATP} channels. Using these surface-selective agents, we investigated whether surfaceK\text{ATP} channels are involved in cardioprotection.

**Methods**

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85-23, revised 1985).

**Flavoprotein Fluorescence and Electrophysiology of Rabbit Ventricular Myocytes**

Ventricular myocytes were isolated from rabbits\(^17\) and cultured on coverslips in M199 with 5% FBS at 37°C. Experiments were performed the next day. Mitochondrial matrix redox state, reported by the fluorescence of FAD-linked enzymes,\(^18\)\(^19\) was used to index mitoK\text{ATP} channel
activity. Cells were superfused with solution containing (in mmol/L) NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, and HEPES 10 (pH 7.4 with NaOH) at room temperature (~22°C). Endogenous flavoprotein fluorescence was excited with a xenon arc lamp with a bandpass filter centered at 480 nm. Emitted fluorescence was recorded from 1 cell at a time at 530 nm by a photomultiplier tube and expressed as a percentage of the DNP-induced fluorescence. In some experiments, flavoprotein fluorescence was measured during whole-cell patch-clamp experiments to administer drugs through the pipette (cf Figure 3).

For whole-cell patch recordings, the internal pipette solution contained (in mmol/L) potassium glutamate 120, KCl 25, MgCl₂ 0.5, K-EGTA 10, HEPES 10, and MgATP 1 (pH 7.2 with KOH). Currents were elicited every 6 seconds from a holding potential of ~80 mV by 2 consecutive steps to ~40 mV (for 100 ms) and 0 mV (for 380 ms). Current amplitude at 0 mV was measured 200 ms into the pulse to quantify surfaceKₐtp channel activity. In some experiments (e.g., Figure 6), whole-cell currents and flavoprotein fluorescence were recorded simultaneously, and flavoprotein fluorescence was excited during the 100-ms step to ~40 mV.

**Functional Expression of Kₐtp Channels and Electrophysiology**

Details of the functional expression of Kₐtp channels in HEK 293 cells have been described previously. Plasmid DNA (3 μg total) containing Kir6.1 or Kir6.2 was cotransfected with either SUR2B or SUR2A cDNA into HEK cells by use of lipofectamine (Gibco) 18 hours after the cells were split. Mouse Kir6.1, provided by Prof Y. Kurachi (Osaka University, Japan), and rabbit Kir6.2 (GenBank AF006262) were cloned into vector pGFP-IRES. Rat SUR2A, supplied by Prof S. Seino (Chiba University, Japan), was expressed in the mammalian vector pCMV6. Mouse SUR2B, supplied by Prof Y. Kurachi, was cloned into the expression vector pCDNA3.

Electrophysiological recordings were made 48 hours after transfection with solutions identical to those used in rabbit ventricular myocytes (see above). Voltage ramps from -100 to +60 mV were applied every 100 ms every 6 seconds from a holding potential of ~80 mV. The current at 0 mV was measured to assay Kₐtp channel activity. Experiments were performed at room temperature (~22°C).

**Simulated Ischemia and Cellular Injury**

A cell-pelleting model of ischemia modified from Vander Heide et al. was used to quantify myocyte injury. In brief, adult rabbit ventricular cells were washed with incubation buffer: (in mmol/L) NaCl 119, NaHCO₃ 25, KH₂PO₄ 1.2, KCl 4.8, MgSO₄ 1.2, CaCl₂ 1, glucose 11, and taurine 58.5, supplemented with 1% HEPES 10, and 0.5% glutaraldehyde and 0.5% trypan blue. Cells were centrifuged for 10 seconds. Approximately 0.25 mL of supernatant was removed to leave a thin fluid layer above the pellet, and 0.2 mL of mineral oil was layered on the top to prevent gaseous diffusion. After 60 minutes or 120 minutes, 5 μL of cell pellet was sampled through the oil layer and mixed with 75 μL of 85 mOsm hypotonic staining solution: (in mmol/L) NaCl 119, NaHCO₃ 11.9, KH₂PO₄ 0.4, KCl 2.7, MgSO₄ 0.8, and CaCl₂ 1, with 0.5% glutaraldehyde and 0.5% trypan blue. Cells permeable to trypan blue were counted and expressed as a percentage of the total cells counted (~300 for each sample).

In the control group, cells were pelleted and sampled at 60 or 120 minutes. For the diazoxide-treated or P-1075–treated groups, diazoxide (100 μmol/L) or P-1075 (30 μmol/L) was added to the incubation buffer 10 minutes before the IPC. All 4 conditions were tested simultaneously in each of 6 replications.

The small percentage of cells (~18%) that were nonviable at the beginning of the experiment were mostly rounded and had been damaged as a consequence of the enzymatic isolation process. The osmotic fragility of cells induced by ischemia was quantified as percentage of the vital cells at the beginning of each experiment. In nonpelleted control cells suspended in oxygenated buffer with or without drugs, there was no change in the percentage of stained cells measured after 120 minutes of incubation. Pelleting experiments were performed at 37°C.

**Chemicals**

Diazoxide and DNP were purchased from Sigma Chemical Co. 5HD was purchased from Research Biochemicals International. HMR1098 was a gift from Hoechst Marion Roussel (now Aventis Pharmaceuticals), and P-1075 was a gift from Leo Pharmaceutical Products. Diazoxide and P-1075 were dissolved in DMSO before being added into the experimental solution. The final concentration of DMSO was <0.1%.

**Statistical Analysis**

All data are presented as mean±SEM, and the number of cells or experiments is shown as n. Statistical analysis was performed with ANOVA combined with the Fisher post hoc test. Values of P<0.05 were considered significant.

**Results**

**Effect of HMR1098 on SurfaceKₐtp and MitoKₐtp Channels**

We first verified the inhibitory effect of HMR1098 on surfaceKₐtp channels by whole-cell patch clamp. Figure 1A shows surfaceKₐtp current (Iₐtp) elicited by exposure to 2.4-dinitrophenol (DNP, 100 μmol/L). Although DNP eventually increased Iₐtp, subsequent application of 30 μmol/L HMR1098 suppressed Iₐtp. As summarized in Figure 1B, HMR1098 (30 μmol/L) inhibited DNP-induced Iₐtp from 2.22±0.89 to 0.52±0.14 nA (P<0.05, n=5).

The effects of HMR1098 on mitoKₐtp channels were examined by measuring mitochondrial matrix redox potential. Figure 2A shows the time course of flavoprotein fluorescence in a cell exposed twice to diazoxide, a selective mitoKₐtp channel opener in heart cells. Diazoxide (100 μmol/L) induced reversible oxidation of the flavoproteins. A second application of diazoxide in the presence of HMR1098...
(30 μmol/L) once again increased the flavoprotein fluorescence, and the degree of oxidation was identical to that achieved during the first exposure to diazoxide. As summarized in Figure 2B, diazoxide (100 μmol/L) reversibly increased flavoprotein oxidation to 41±8% of the DNP value (n=4). HMR1098 (30 μmol/L) did not alter the effect of diazoxide (42±9% of the DNP value, n=4).

To verify that the lack of effect of HMR1098 on diazoxide-induced flavoprotein oxidation did not result from inadequate diffusion of the drug to mitochondria, we measured flavoprotein fluorescence after including HMR1098 (30 μmol/L) in the patch pipette. Figure 3A shows that after 10 minutes in the whole-cell configuration, exposure to diazoxide (100 μmol/L) still induced flavoprotein oxidation. This effect of diazoxide could be blocked by 5HD (500 μmol/L), a specific mitoKATP channel inhibitor.10,11 Subsequent reapplication of diazoxide in the absence of 5HD once again increased flavoprotein oxidation. Despite the presence of HMR1098 in the pipette, diazoxide increased flavoprotein oxidation to 46±6% of the DNP value (n=5, Figure 3B). This degree of oxidation is comparable to that observed in the absence of HMR1098 (cf Figure 2). The results indicate that HMR1098 selectively inhibits surfaceKATP channels but not mitoKATP channels.

Effect of P-1075 on SurfaceKATP and MitoKATP Channels

We then examined the effects of the KATP channel opener P-107516 on surfaceKATP and mitoKATP channels. P-1075 is a derivative of the cyanoguanidine KATP channel agonist pinacidil, which is known to open both mitoKATP and surfaceKATP channels.8 Figure 4, A and B, shows that P-1075 (30 μmol/L) significantly increased \( I_{\text{K,ATP}} \) (P<0.01 vs control (CONT) and P-1075+HMR1098. C, Dose-response curve for P-1075 in rabbit ventricular myocytes. Each point constitutes measurements from 4 to 5 cells. D, Dose-dependent effects of P-1075 on Kir6.2+SUR2A (cardiac-type) and Kir6.1+SUR2B KATP (vascular smooth muscle–type) channels expressed in HEK cells. Each point constitutes measurements from 5 to 10 cells.
Kir6.1 + SUR2B (vascular smooth muscle type)\textsuperscript{23} K\textsubscript{ATP} channels. In cells expressing the cardiac-type Kir6.2 + SUR2A channels, P-1075 effectively increased $I_{\text{K,ATP}}$ in a concentration-dependent manner (EC\textsubscript{50} for P-1075 = 2.5 \mu mol/L). Conversely, P-1075 activated Kir6.1 + SUR2B K\textsubscript{ATP} channels at nanomolar concentrations (EC\textsubscript{50} = 102 \mu mol/L). Thus, the low-dose effects previously described\textsuperscript{24} are unlikely to reflect activation of cardiac surface K\textsubscript{ATP} channels.

The effects of P-1075 on mitoK\textsubscript{ATP} channels were examined by measurement of mitochondrial redox potential. Figure 5 shows that diazoxide (100 \mu mol/L) induced reversible oxidation of the mitochondrial matrix to 44\% of the DNP value (n = 5). Subsequent exposure to P-1075 (30 \mu mol/L) had no effect (2\% of the DNP value, n = 5), whereas diazoxide once again increased flavoprotein oxidation (39\% of the DNP value, n = 4). Even when very high (100 \mu mol/L) or low (10 \mu mol/L) concentrations of P-1075 were applied, the drug failed to elicit any flavoprotein response (not shown). These results indicate that P-1075 selectively activates surfaceK\textsubscript{ATP} channels without affecting mitoK\textsubscript{ATP} channels.

To verify further the specificity of diazoxide and P-1075 for mitoK\textsubscript{ATP} and surfaceK\textsubscript{ATP} channels, respectively, we measured flavoprotein fluorescence and membrane current simultaneously. Figure 6A and 6B shows the effects of diazoxide and P-1075 in a representative experiment. Diazoxide (100 \mu mol/L) induced reversible oxidation of flavoproteins but did not affect $I_{\text{K,ATP}}$. In contrast, exposure to P-1075 (30 \mu mol/L) failed to increase flavoprotein oxidation but did elicit $I_{\text{K,ATP}}$. As summarized in Figure 6C and 6D, unlike diazoxide, P-1075 activated only $I_{\text{K,ATP}}$.

**Effects of HMR1098 and P-1075 on Simulated Ischemia and Cellular Injury**

Using the mitochondria- or surface-selective agents, we examined the role of mitoK\textsubscript{ATP} and surfaceK\textsubscript{ATP} channels for ischemic cardioprotection. The mitoK\textsubscript{ATP} channel opener diazoxide (100 \mu mol/L) significantly decreased the percentage of cells stained after 60 minutes of simulated ischemia (from 32\% to 17\%, $P<0.001$, n = 5), and this protection was completely prevented by the mitoK\textsubscript{ATP} channel blocker 5HD (500 \mu mol/L) (Figure 7A). In contrast, the surfaceK\textsubscript{ATP} channel blocker HMR1098 (30 \mu mol/L) did not prevent the cardioprotection by diazoxide (from 38\% to 18\%, $P<0.001$, n = 4) (Figure 7B). In a separate series of experiments (Figure 7C), simulated ischemia for 60 and 120 minutes stained 35\% (n = 5) and 42\% (n = 5) of cells, respectively. Inclusion of diazoxide (100 \mu mol/L) significantly decreased the percentage of cells stained, to 18\%.
(n=5) after 60 minutes and 24±2% (n=5) after 120 minutes of simulated ischemia (P<0.001 versus control group). In contrast, the selective surfaceKATP channel opener P-1075 (30 μmol/L) did not alter the extent of stained cells as a consequence of ischemia (34±2% after 60 minutes, 38±4% after 120 minutes). These results indicate that mitoKATP but not surfaceKATP channels are involved in pharmacological cardioprotection.

We examined the effects of 5HD and HMR1098 on simulated IPC. As summarized in Figure 8, IPC significantly decreased the percentage of cells stained during 60 minutes (from 26±4% to 13±2%, P<0.005, n=6) and 120 minutes (from 35±3% to 20±3%, P<0.005, n=6) of simulated ischemia. 5HD (500 μmol/L) added 10 minutes before preconditioning abolished the protection (25±3% after 60 minutes and 34±3% after 120 minutes ischemia, respectively). In contrast, HMR1098 (30 μmol/L) did not interfere with IPC after 60 minutes (15±2%, P<0.005, n=6) or 120 minutes (20±3%, P<0.005, n=6) of simulated ischemia. These results indicate that the cardioprotection afforded by IPC is mediated by mitoKATP channels, not surfaceKATP channels.

Discussion

The cardioprotective effects of IPC are abolished by antagonists of KATP channels and are mimicked by agonists of KATP channels. On the basis of these studies, an early hypothesis proposed that opening of surfaceKATP channels abbreviates action potential duration, thereby reducing cellular calcium overload and preserving viability in ischemic myocardium. However, this hypothesis cannot account for IPC, because abbreviation of action potential duration is not necessary for protection. Grover et al showed that dofetilide attenuated the shortening of action potential duration but did not abolish IPC in dogs. Yao and Gross demonstrated that low-dose bimakalim produced cardioprotection without any effect on monophasic action potential duration. Furthermore, IPC occurs even in unstimulated (electrically quiescent) cardiac myocytes, in which action potential abbreviation cannot be a factor.

MitoKATP channels share some pharmacological properties with surfaceKATP channels, while possessing a distinct pharmacological profile. Garlid et al demonstrated that diazoxide opens mitoKATP channels >2000-fold more potently than surfaceKATP channels in cardiac myocytes. Although direct effects of diazoxide on mitochondrial energy metabolism in pancreatic β-cells have been proposed, previous studies in our laboratory demonstrated that diazoxide oxidizes the mitochondrial matrix redox potential via opening of mitoKATP channels in rabbit hearts, whereas surfaceKATP channels are quite resistant to this drug; the specificity of diazoxide for mitoKATP channels is verified in Figure 6 of the present article. Moreover, we reported that 5HD completely and reversibly blocks the oxidative effect of diazoxide without affecting surfaceKATP channels, indicating that 5HD is an effective and specific blocker of mitoKATP channels (although this may not be the case in other species). When these drugs are used as pharmacological tools to activate or to inhibit mitoKATP channels, growing evidence supports the idea that mitoKATP channels rather than surfaceKATP channels are the dominant players for cardioprotection. Moreover, in animal models in vivo, diazoxide mimics whereas 5HD abolishes the infarct size-limiting effect of IPC. These findings motivated us to reevaluate the role of surfaceKATP channels in cardioprotection.

The sulfonylthiourea HMR1098 has been reported to be a cardioselective KATP channel blocker, blocking KATP channels in cardiac muscle cells with 10- to 50-fold higher potency than in pancreatic β-cells with little effect on the coronary vasculature. We demonstrated that HMR1098 inhibited surfaceKATP channels activated by metabolic inhibition (Figure 1) and by the surfaceKATP channel opener P-1075 (Figure 4). However, HMR1098 did not affect diazoxide-induced flavoprotein oxidation (Figure 2). Furthermore, direct application of HMR1098 to the cytoplasm by inclusion in the pipette failed to prevent the oxidizing effect of diazoxide (Figure 3). These results, taken together, indicate that HMR1098 selectively inhibits surfaceKATP channels without affecting mitoKATP channels.

The KATP channel opener P-1075 activated IATP in rabbit ventricular myocytes; IATP was blocked by HMR1098 (Figure 4). Moreover, the molecularly defined cardiac surfaceKATP channel (Kir6.2+SUR2A) expressed in HEK cells was effectively activated by P-1075. Indeed, P-1075 showed similar potencies in activating native surfaceKATP channels and expressed Kir6.2+SUR2A channels (EC50=8 of 13 μmol/L and 2.5 μmol/L, respectively). P-1075 activated Kir6.1+SUR2B (vascular smooth muscle type) channels ≈100-fold more potently than Kir6.2+SUR2A channels (EC50 of 102 nmol/L). Conversely, P-1075 did not affect mitochondrial oxidation state measured with or without invasion by patch pipettes or their contents (Figures 5 and 6). These results indicate that, unlike diazoxide, P-1075 selectively activates surfaceKATP but not mitoKATP channels.

We previously reported that diazoxide (50 μmol/L) decreased myocyte death in a cellular model of simulated ischemia. In the present study, we verified the cardioprotective effects of diazoxide (Figure 7). Diazoxide-induced cardioprotection was prevented by the mitoKATP channel blocker 5HD. In contrast, the surfaceKATP channel blocker HMR1098 did not abolish the cardioprotective effect of diazoxide (Figure 7B). Furthermore, we found that the surfaceKATP channel opener P-1075 did not

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Figure 8. Effects of 5HD and HMR1098 on ischemically preconditioned cells. Cells stained after 60 and 120 minutes of simulated ischemia were plotted as percentage of total viable cells before ischemia. CONT indicates nonpreconditioned control group; IPC, preconditioned group; IPC+5HD, 5HD (500 μmol/L) added 10 minutes before preconditioning; and IPC+HMR1098, HMR1098 (30 μmol/L) added 10 minutes before preconditioning. *P<0.005 vs CONT group.
produce cardioprotection (Figure 7C). Sargent et al.\(^1\) reported that, in Langendorff-perfused rat hearts, P-1075 increases coronary flow and protects ischemic myocardium at nanomolar concentrations. Consistent with their findings, P-1075 does activate the vascular smooth muscle type (Kir6.1+SUR2B) K\(_{ATP}\) channel at nanomolar concentrations, whereas the cardiac-type (Kir6.2+SUR2A) K\(_{ATP}\) channel is affected only in the micromolar range (Figure 4D). However, we found that the surface K\(_{ATP}\) channel opener P-1075 (30 \(\mumol/L\)) did not produce cardioprotection (Figure 7C). Although the reason for this discrepancy is unknown, our results imply that the direct activation of surface K\(_{ATP}\) channels in rabbit myocytes does not underlie myocyte cardioprotection.

IPC is present in all species examined, including humans.\(^3\) Compelling evidence suggests that mitoK\(_{ATP}\) channels rather than surface K\(_{ATP}\) channels may serve as end effectors of IPC. However, another study reported that digoxin preserves the subsarcolemmal ATP and inhibits surface K\(_{ATP}\) channels, thereby abolishing IPC in rabbit hearts.\(^3\) To clarify these discordant data, further studies using the selective agonist and antagonist of surface K\(_{ATP}\) or mitoK\(_{ATP}\) channels are necessary. Our present results demonstrate that the mitoK\(_{ATP}\) channel blocker 5HD but not the surface K\(_{ATP}\) channel blocker HMR1098 abolished the cardioprotection in a cellular model of preconditioning. These results resolve the cardioprotective effect of IPC from activation of surface K\(_{ATP}\) channels.

In conclusion, our observations with diazoxide, P-1075, 5HD, and HMR1098 provide the first definitive pharmacological evidence that surface K\(_{ATP}\) channels are not involved in cardioprotection in isolated rabbit myocytes. By extension, the present data provide further support for the emerging consensus that mitoK\(_{ATP}\) channels are the end effectors of preconditioning specifically in rabbit heart.

Acknowledgments

This study was supported by the National Institutes of Health (R37-HL-36957 to Dr Marbán) and by a Banyu Fellowship in Lipid Metabolism and Atherosclerosis (to Dr Sato). Dr Marbán holds the Banyu Fellowship in Lipid Metabolism and Atherosclerosis (R37-HL-36957 to Dr Marbán) and by a Banyu Fellowship in Lipid Metabolism and Atherosclerosis.

Our present results demonstrate that the mitoK\(_{ATP}\) channel rather than surface K\(_{ATP}\) channels are involved in cardioprotection in isolated rabbit myocytes. By extension, our results imply that the direct activation of surfaceK\(_{ATP}\) channels in rabbit myocytes does not underlie myocyte cardioprotection.

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_Circulation_. 2000;101:2418-2423
doi: 10.1161/01.CIR.101.20.2418

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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