Additive Effects of Late Preconditioning Produced By Monophosphoryl Lipid A and the Early Preconditioning Mediated By Adenosine Receptors and $K_{\text{ATP}}$ Channel

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**Background**—The cardioprotective effect of preconditioning can be exerted within 1 to 2 hours after initial ischemia, termed classical or early preconditioning, or can reappear 24 hours later as second window or late preconditioning. The objective of this study was to study the interaction between late and early preconditioning and to determine the potential underlying mechanism.

**Methods and Results**—Adenosine receptor agonists and a $K_{\text{ATP}}$ channel opener were used to achieve early preconditioning, and Monophosphoryl lipid A (MLA) was used to induce late preconditioning. Cultured chick ventricular myocytes were used as a myocyte model of simulated ischemia and preconditioning. Prior treatment of the myocyte with MLA caused a dose-dependent decrease in the ischemia-induced myocyte injury 24 hours later, consistent with a late preconditioning effect. L-NMMA, glibenclamide, or 5-hydroxydecanoic acid administered during the ischemia blocked the MLA effect. Twenty four hours after MLA treatment, a 5-minute exposure to ischemia, adenosine, adenosine $A_1$ agonist CCPA, or $A_3$ agonist resulted in less myocyte injury during the subsequent prolonged ischemia, as compared with cells pretreated with the vehicle and subsequently exposed to the same early preconditioning stimuli. In addition to its ability to enhance the early preconditioning effect by $A_1$ and $A_3$ agonists, MLA pretreatment also increased the phorbol ester- and pinacidil-mediated early preconditioning effect.

**Conclusions**—This study defined a novel interaction in which the cardioprotective effect of early preconditioning is additive to that of late preconditioning and raised the possibility that both agents can be used as combined therapy in the treatment of ischemic heart disease. (*Circulation*. 1999;99:3300-3307.)

**Key Words:** myocardium ■ receptors ■ hypoxia ■ ions
Methods
Preparation of Cultured Ventricular Myocytes
Ventricular myocytes were cultured from chick embryos 14 days in ovo according to a previously described procedure.\textsuperscript{15} Myocytes were maintained in culture medium containing 6\% fetal bovine serum, 40\% medium 199 (GIBCO), 0.1\% penicillin/streptomycin, and a salt solution. The final concentrations in the culture medium were Na\textsuperscript{+} 142, K\textsuperscript{+} 3.3, Mg\textsuperscript{2+} 0.7, Ca\textsuperscript{2+} 1.4, Cl\textsuperscript{−} 110, HCO\textsubscript{3}− 14.4, and glucose 5.5 mmol/L, respectively. Myocytes were cultivated in a humidified 5\% CO\textsubscript{2}-95\% air mix at 37°C and allowed to grow to a monolayer on day 3, at which time they exhibited rhythmic, spontaneous contraction. All experiments were performed on cells at day 3 in culture.

Simulation of Ischemia and Preconditioning of Cardiac Myocytes
On day 3 of cultivation, the medium was changed to a N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-buffered medium containing 139 mmol/L NaCl, 4.7 mmol/L KCl, 0.5 mmol/L MgCl\textsubscript{2}, 0.9 mmol/L CaCl\textsubscript{2}, 5 mmol/L HEPES, and 2\% fetal bovine serum, pH 7.4, before exposing the myocytes to the various conditions at 37°C. Cultured myocytes were maintained in the HEPES-buffered media under room air (normal percentage of oxygen). Ninety-minute exposure of the myocytes to hypoxia with glucose deprivation was used to simulate ischemia and induce cell injury. Hypoxia was produced by placing the cells in a hypoxic incubator (NuAire) where O\textsubscript{2} was replaced by N\textsubscript{2}, as previously described.\textsuperscript{15,16} Effects of adenosine receptor agonists and a K\textsubscript{ATP} channel opener on the extent of myocyte injury were determined by exposure of the cells to these agents during the prolonged simulated ischemia.

Preconditioning of the cultured cardiac ventricular myocytes was performed according to a previously established procedure.\textsuperscript{15,16} Briefly, before preconditioning, the culture medium was replaced with a HEPES-buffered medium containing 139 mmol/L NaCl, 4.7 mmol/L KCl, 0.5 mmol/L MgCl\textsubscript{2}, 0.9 mmol/L CaCl\textsubscript{2}, 5 mmol/L HEPES and 2\% fetal bovine serum, pH = 7.4, at 37°C. Hypoxia was produced by placing the myocytes in a hypoxic incubator (NuAire) where O\textsubscript{2} was replaced by N\textsubscript{2}, as previously described.\textsuperscript{15,16} To induce early preconditioning by pharmacological means, myocytes were treated with the phosphor ester PMA (phorbol 12-myristate 13-acetate), 2-chloro-N\textsuperscript{6}-cyclopentyladenosine (CCPA), N\textsuperscript{6}-(3-iodobenzyl)adenosine-5’-N\textsuperscript{6}-methyluronamide (IB-MECA), or pinacidil for 5 minutes instead of being exposed to the brief simulated ischemia. Media containing the protein kinase C (PKC) activator PMA, adenosine analog CCPA, IB-MECA, or pinacidil were then replaced with fresh media lacking the agent. Myocytes thus pretreated were then exposed to 90 minutes of hypoxia. To induce late preconditioning, myocytes were exposed to the vehicle (0.01\% ethanol and 0.04\% propylene glycol) or to varying concentrations of MLA dissolved in the same vehicle for 4 hours. Media were then replaced with vehicle- or MLA-free media until further interventions 24 hours later. The various interventions include studies on the early preconditioning response mediated by CCPA, IB-MECA, PMA, or pinacidil and the cardioprotective efficacy of each of these agents during the sustained ischemia.

Myocytes not subjected to preconditioning were exposed to 90 minutes of hypoxia only (non preconditioned). Control myocytes were maintained in the HEPES-buffered media at 37°C under room air. Determination of basal level of myocyte injury was made after parallel incubation of control myocytes without exposure to simulated ischemia. The determination of myocyte injury was made at the end of the 90-minute ischemic period, at which time the cells were removed from the hypoxic incubator and reexposed to room air. Aliquots of the medium were then obtained for creatine kinase (CK) activity measurement, which was followed by quantification of the number of viable cells.

For preconditioned and nonpreconditioned myocytes, the extent of myocyte injury was determined by the amount of CK released into the media and by the percentage of myocytes killed. The amount of CK was measured as enzyme activity (in U per milligram), and increases in CK activity above the control level were plotted. The release of CK correlated with that of proteins and lactate dehydrogenase in the media.\textsuperscript{15,16} The percentage of cells killed was calculated as the number of cells obtained from the control group (representing cells not subjected to any hypoxia or drug treatment) minus the number of cells from the treatment group, divided by the number of cells in the control group×100\%. Prior studies have shown that both methods represent independent means of quantifying the extent of myocyte injury.\textsuperscript{15,16} Differences in the percentages of cells killed or the amount of CK released were analyzed by 1-way ANOVA followed by post-test comparison (Student-Newman-Keuls multiple comparison test).

Results
Cardioprotective Effect of MLA in Cardiac Myocytes: A Late Preconditioning Effect
Prior treatment of the cardiac myocytes with 30 ng/mL and 300 ng/mL of MLA decreased the number of cardiac cells killed during a prolonged period of ischemia 24 hours later (Figure 2). The percentage of cells killed following MLA treatment was significantly less than that following vehicle treatment (1-way ANOVA followed by Student-Newman-Keuls multiple comparison test, \textit{P}<0.01). Thus, MLA is capable of causing a second window of protection or a late preconditioning effect in these cultured cardiac myocytes. This second window of protection was abolished by 100 \textmu mol/L glibenclamide or 5-HD added to the media during the prolonged ischemia (Figure 3). At 10- or 30-fold lower concentrations of either K\textsubscript{ATP} channel blocker, there was significant inhibition of the MLA-induced cardioprotective effect (data not shown). These data are consistent with a role of K\textsubscript{ATP} channel in mediating the late preconditioning effect of MLA, similar to those reported by others.\textsuperscript{6–9} To examine the role of NO in mediating the late preconditioning effect of MLA, the nonselective nitric oxide synthase (NOS) inhibitor L-NMMA was added during the prolonged ischemia 24 hours after the exposure to MLA. Figure 4 shows that L-NMMA blocked the late preconditioning effect induced by MLA.

MLA Enhanced Early Preconditioning Effect Elicited By Adenosine and Adenosine Receptor Agonists
Twenty four hours after MLA exposure, the ability of adenosine, adenosine A\textsubscript{1} receptor agonist CCPA, A\textsubscript{1} receptor agonist IB-MECA, or brief ischemia to induce early preconditioning was determined. Prior treatment with MLA caused an enhanced adenosine- or ischemia-mediated early or classic
preconditioning effect. A 5-minute exposure to adenosine (10 μmol/L) resulted in significantly fewer cells killed during the sustained ischemia in MLA-treated myocytes (percentage of cells killed = 7 ± 1 (± SE), n = 5) compared with vehicle-treated cardiac myocytes (11 ± 2, P < 0.05, t test). Similarly, a 5-minute exposure to simulated ischemia led to fewer cells killed (Figure 2) and less CK released (not shown) during the subsequent 90-minute ischemia in myocytes pretreated with MLA, compared with those pretreated with the vehicle. Thus, the late preconditioning effect of MLA is additive with the early effect of ischemic preconditioning.

Because activation of the adenosine A<sub>1</sub> and A<sub>3</sub> receptors mediates the early preconditioning effect of adenosine, the role of MLA in enhancing the A<sub>1</sub> or A<sub>3</sub> agonist-induced early preconditioning response was also determined. In MLA-pretreated myocytes, a 5-minute exposure to CCPA or IB-MECA led to a decreased number of cells killed during a subsequent 90-minute period of ischemia in comparison with cells not pretreated with MLA (Figure 5A and 5B). Thus, MLA enhanced the ability of both adenosine A<sub>1</sub> and A<sub>3</sub> receptors to mediate the early preconditioning response.

Effects of MLA on Early Preconditioning Response Mediated By PMA and Pinacidil

The K<sub>ATP</sub> channel is a downstream effector of PKC, which in turn acts consequent to adenosine receptor activation in mediating the early preconditioning response in the cardiac myocyte. Therefore, the effects of MLA on the early preconditioning responses induced by the PKC activator PMA and the K<sub>ATP</sub> channel opener pinacidil were determined. Prior treatment with MLA increased the ability of PMA and pinacidil to cause early preconditioning of the cardiac myocytes. In MLA-treated myocytes, compared with myocytes treated with the vehicle, there were fewer cells killed during the prolonged ischemia following a 5-minute exposure to PMA or pinacidil (Figure 6A and 6B).

Effects of Prior MLA Treatment on Subsequent Ability of CCPA, IB-MECA, or Pinacidil to Protect Myocyte During Sustained Ischemia

Previous study in this cultured myocyte model demonstrated that CCPA or IB-MECA exerts a potent cardioprotective effect even when ischemia has begun. Because MLA...
enhanced the ability of CCPA and IB-MECA to induce the early preconditioning response, MLA may also enhance the A1 or A3 agonist-mediated cardioprotection during the sustained ischemia. In these studies, myocytes were pretreated with MLA or the vehicle. Twenty four hours later, myocytes were exposed to sustained ischemia while simultaneously incubated in the presence of CCPA, IB-MECA, or pinacidil. Prior treatment of the cardiac myocyte with MLA, compared with myocytes pretreated with the vehicle, resulted in significantly fewer cells killed in the presence of CCPA, IB-MECA, or pinacidil during the 90-minute ischemia (Figure 7A through C). The ability of each agent to protect against ischemia-induced myocyte injury was evident at even the lowest concentrations tested.

Discussion
A brief episode of ischemia before a second, sustained period of ischemia can protect the myocardium against infarction, a phenomenon known as ischemic preconditioning. Two types of preconditioning have been described. In early or classic preconditioning, the cardioprotective effect lasts 1 to 2 hours after the preconditioning stimulus. In late preconditioning, the cardioprotective effect reappears 24 hours after the preconditioning stimulus and is also termed the second window of protection. Both types of preconditioning can be induced by exposure to brief ischemia or adenosine receptor agonists. Although both types of preconditioning can confer cardioprotection, interaction between the 2 preconditioning processes and the potential underlying mechanism remain unknown. Therefore, the objective of this study was to investigate such interaction and the mechanism involved. In...
the study, MLA was shown to induce late preconditioning and adenosine receptor agonists were used to cause the early preconditioning.

Consistent with prior studies, MLA was also able to cause a potent cardioprotective effect 24 hours after the exposure of the cultured cardiac myocytes to MLA. Blockade of cardioprotection by the K<sub>ATP</sub> channel antagonists glibenclamide and 5-HD suggested a key role of the channel in mediating MLA-induced protection, similar to the findings in intact canine heart and in adult rat cardiac myocytes. These data are consistent with the current hypothesis on the mechanism by which MLA induces late preconditioning. The concentration of glibenclamide and 5-HD used in the current study was high (100 μmol/L); at this concentration, glibenclamide will also block the cystic fibrosis conductance regulator and swelling-activated and calcium-activated chloride channels. However, 4 additional lines of evidence support a role of the channel in mediating the cardioprotective effect of MLA. First, glibenclamide and 5-HD, at 100 μmol/L, were able to block the protective effect of the K<sub>ATP</sub> channel opener pinacidil during prolonged ischemia, indicating successful inhibition of the channel. Second, if blockade of the various chloride channels worsened the myocyte injury, glibenclamide or 5-HD alone would have increased the ischemia-induced injury. However, this was not the case (data not shown). Third, MLA treatment enhanced the ability of pinacidil to precondition the myocytes and to protect the myocytes against injury during the sustained ischemia. Fourth, the NO inhibitor L-NMMA was able to block the late preconditioning effect induced by MLA. NO has been suggested to enhance the open state probability of cardiac or vascular smooth muscle K<sub>ATP</sub> channels and to mediate the cardioprotective effect of late preconditioning. The ability of the NO donor S-nitrosoglutathione to protect these cultured myocytes during the 90-minute ischemia was abolished by glibenclamide or 5-HD (data not shown). Taken together, these data provide further evidence for the hypothesis that MLA-induced late preconditioning is mediated by an NO-dependent activation of the K<sub>ATP</sub> channel.

Figure 5. Effects of prior MLA treatment on early preconditioning response to adenosine, adenosine receptor agonists. Cardiac ventricular myocytes were prepared and preexposed to 300 ng/mL of MLA or to vehicle as described in Figure 2. Myocytes were subjected to early preconditioning protocol outlined in Figure 1B 24 hours later. In brief, the myocytes were exposed to CCPA (A) or IB-MECA (B) for 5 minutes, followed by incubation in adenosine- or agonist-free media for 10 minutes before 90 minutes of ischemia. Data, plotted as percentage of cells killed and the amount of CK released, represent the mean±SE of 4 experiments. *Significantly different from vehicle-treated myocytes at concentrations of adenosine agonists indicated (t test, P<0.05).
Previous studies indicated that adenosine receptor activation can induce an early preconditioning response in cardiac myocytes. The late preconditioning effect induced by MLA is additive with the cardioprotective effect of early preconditioning induced by adenosine or a brief exposure to ischemia. Thus, 24 hours following MLA treatment, adenosine or brief ischemia caused a more pronounced preconditioning effect compared with vehicle treatment of the myocytes. Because activation of the adenosine $A_1$ and $A_3$ receptors mediates the preconditioning effect of adenosine, the effect of prior MLA treatment on the CCPA- and IB-MECA-mediated preconditioning effect was determined. MLA was able to enhance the early preconditioning response mediated by either CCPA or IB-MECA. Together, these data demonstrated a novel, productive interaction between the early and the late preconditioning.

Because a sequential activation of the adenosine receptor, PKC, and $K_{ATP}$ channel mediates the early preconditioning response to adenosine, the mechanism by which MLA enhances the adenosine receptor-mediated preconditioning response may be exerted at the level of the receptor, PKC, or the $K_{ATP}$ channel. If MLA acts to activate the receptor only, MLA would not enhance the maximal PMA- or pinacidil-induced early preconditioning response. If MLA activates PKC directly, MLA would enhance the receptor- and PKC-mediated preconditioning response but not the maximal $K_{ATP}$ channel-mediated preconditioning response. However, if MLA activates the $K_{ATP}$ channel, MLA should increase the preconditioning effect mediated by all 3. The present data showed that MLA pretreatment can indeed enhance the preconditioning effect mediated by adenosine receptors, PKC, and the $K_{ATP}$ channel. Although it is possible that MLA can activate all 3 proteins directly, a simultaneous activation is less likely. The most parsimonious explanation of the present findings is that MLA activates the $K_{ATP}$ channel and renders the channel more responsive to an adenosine agonist, PKC activator, or $K_{ATP}$ channel opener. Alternatively, if maximally effective concentrations of adenosine receptor

Figure 6. Effects of prior MLA treatment on early preconditioning response to PMA and pinacidil. Cardiac ventricular myocytes were prepared and preexposed to 300 ng/mL of MLA or to vehicle as described in legend to Figure 2. Myocytes were subjected to early preconditioning protocol outlined in Figure 1B 24 hours later. PMA (A) or pinacidil (B), at the indicated concentrations, were used as early preconditioning stimulus. Data, plotted as percentage of cells killed and the amount of CK released, represent mean±SE of 4 experiments. *Significantly different from vehicle-treated myocytes at concentrations of PMA or pinacidil indicated (t test, $P<0.05$).
Figure 7. MLA treatment can enhance ability of CCPA, IB-MECA, and pinacidil to protect cardiac myocytes during sustained ischemia. Cardiac ventricular myocytes were prepared and preexposed to 300 ng/mL of MLA or vehicle as described in Figure 1A. Myocytes were then exposed to 90 minutes of simulated ischemia in the presence of various concentrations of CCPA (A), IB-MECA (B), or pinacidil (C). Data represent mean±SE of 4 experiments. *Significantly different from vehicle-treated myocytes at the concentrations indicated (t test, \( P < 0.05 \)).
agonists, PKC activator, and K\textsubscript{ATP} channel opener were not used, MLA could enhance the cardioprotection by an additive mechanism by which the protective effect of a sub-maximal concentration of one agent could be improved by using a sub-maximal concentration of a second agent.

To provide further evidence for this notion, the ability of MLA to enhance the CCPA-, IB-MECA-, or pinacidil-mediated cardioprotection during the sustained ischemia was examined. Previous study has shown a potent, protective effect of CCPA and IB-MECA when each agonist is individually present during the sustained ischemia.\textsuperscript{17} K\textsubscript{ATP} channel is downstream of the adenosine receptor in mediating this protective effect during the sustained ischemia.\textsuperscript{18} If MLA acts to activate the K\textsubscript{ATP} channel, MLA should then enhance the cardioprotective effect of not only pinacidil, but also CCPA and IB-MECA. The present data indicate that this is in fact the case. Prior treatment with MLA led to an enhanced protective effect of pinacidil, CCPA, or IB-MECA during the sustained ischemia. Together, the data provide further evidence for the concept that the mechanism underlying the productive interaction between MLA and the adenosine receptor pathway occurs at the level of the K\textsubscript{ATP} channel. An MLA-activated K\textsubscript{ATP} channel renders the cardiac myocyte more responsive to stimulation by an adenosine A\textsubscript{1} or A\textsubscript{3} receptor agonist and by a K\textsubscript{ATP} channel opener. The positive interaction between the late and the early preconditioning raises the possibility that agents capable of inducing both types of preconditioning can be used as combined therapy in the treatment of ischemic heart disease.

Acknowledgments
This work was supported by RO1-HL48225 from the National Institutes of Health, an Established Investigatorship from the American Heart Association, and a grant from RIBI Immunocinem Research, Inc (to Dr B.T. Liang).

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_Circulation_. 1999;99:3300-3307
doi: 10.1161/01.CIR.99.25.3300

_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

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