Wortmannin, a Potent Antineutrophil Agent, Exerts Cardioprotective Effects in Myocardial Ischemia/Reperfusion

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ABSTRACT

Ischemia followed by reperfusion in the presence of polymorphonuclear leukocytes (PMNs) results in a marked cardiac contractile dysfunction. Wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, suppresses superoxide production from PMNs. Therefore, we hypothesized that wortmannin could attenuate PMN-induced cardiac dysfunction by suppression of superoxide production from PMNs. We examined the effects of wortmannin in isolated ischemic (20 min) and reperfused (45 min) rat hearts perfused with PMNs. Wortmannin at 10, 20, or 40 nM given to hearts during the first 5 min of reperfusion, significantly improved left ventricular developed pressure (P < .01), and the maximal rate of development of left ventricular developed pressure (P < .01) compared with ischemic/reperfused hearts perfused with PMNs in the absence of wortmannin. In addition, wortmannin significantly reduced PMN infiltration into the myocardium by 50 to 75% (P < .001). Superoxide radical release also was significantly reduced in N-formylmethionyl-leucyl-phenylalanine-stimulated PMNs pretreated with 10 or 40 nM wortmannin by 70 and 95%, respectively (P < .001 versus untreated PMNs). Rat PMN adherence to rat superior mesenteric artery endothelium exposed to 2 U/ml thrombin was significantly attenuated by 10 to 40 nM wortmannin compared with untreated vessels (P < .001). These results provide evidence that wortmannin can significantly attenuate PMN-induced cardiac contractile dysfunction in the ischemic/reperfused rat heart via attenuation of PMN infiltration into the myocardium and suppression of superoxide release by PMNs.

Myocardial ischemia followed by reperfusion results in a marked degree of cardiac contractile dysfunction and myocardial cell injury (Forman et al., 1989; Tsao et al., 1990; Lefer and Lefer, 1996). The sequential events that produce this cardiac dysfunction include a decreased endothelial release of nitric oxide (NO), up-regulation of adhesion molecules on the endothelial surface leading to enhanced leukocyte-endothelium interaction, infiltration of polymorphonuclear leukocytes (PMNs) into the myocardium, and subsequent PMN release of superoxide radicals (i.e., the oxidative burst). These radicals are largely responsible for producing cardiac dysfunction and enhanced necrosis (Tsao and Lefer, 1990; Entman et al., 1992; Lefer and Lefer, 1996). The time course of these events starts 2.5 to 5 min postreperfusion with an abrupt decrease in basal release of endothelium-derived NO. PMNs start to transmigrate from the coronary vasculature and infiltrate into the cardiac tissue at 20 min postreperfusion (Tsao and Lefer, 1990; Weyrich et al., 1995; Lefer and Lefer, 1996).

PMNs induce endothelium and myocardial injury by releasing cytotoxic substances such as oxygen-derived free radicals, inflammatory cytokines, and proteolytic enzymes (Forman et al., 1989; Buerke et al., 1994; Hansen, 1995). Previous studies have shown that administration of recombinant human superoxide dismutase (hSOD) can attenuate coronary endothelial and cardiac contractile dysfunction in isolated perfused hearts (Gillespie et al., 1986; Semb et al., 1989; Hansen, 1995). Superoxide radicals contribute to coronary endothelial dysfunction by inactivation of endothelium-derived NO (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986; Patel et al., 1991). Preservation of basal NO release from the coronary endothelium can minimize PMN adherence to the coronary endothelium, leading to preservation of cardiac contractile function in reperfusion injury (Ma et al., 1993; Lefer and Lefer, 1996). In contrast, decreased basal release of NO promotes PMN adherence to the coronary endothelium and subsequent transmigration into inflamed tissue.

ABBREVIATIONS: NO, nitric oxide; PMN, polymorphonuclear leukocyte; hSOD, human recombinant superoxide dismutase; I/R, ischemia/reperfusion; PI3-kinase, phosphatidylinositol 3-kinase; -dp/dt max, maximal rate of development of left ventricular developed pressure; LVDP, left ventricular developed pressure; SMA, superior mesenteric artery; fMLP, N-formyl-methionyl-leucyl-phenylalanine; PtdIns, phosphatidylinositol; PKC, protein kinase C; PI3Kγ, PI3-kinase γ; PLC, phospholipase C.

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isolated perfused rat heart after PMN-induced I/R injury, 2) effect of wortmannin on cardiac contractile function in the presence of PMNs.

The purposes of the present study were to 1) examine the effect of wortmannin on cardiac contractile function in the isolated perfused rat heart after PMN-induced I/R injury, 2) establish the dose-response relationship of wortmannin in this setting, and 3) investigate the mechanism of any observed cardioprotective effect of wortmannin.

Materials and Methods

Isolated Rat Heart Preparation. Male Sprague-Dawley rats (275–325 g) were anesthetized with 60 mg/kg sodium pentobarbital i.p. Sodium heparin (1000 U) also was administered i.p. Hearts were rapidly excised, the ascending aorta was cannulated, and retrograde perfusion of the heart was initiated with a modified Krebs’ buffer maintained at 37°C and at a constant pressure of 80 mm Hg. The Krebs’ buffer had the following composition: 17 mmol/l glucose, 120 mmol/l NaCl, 25 mmol/l NaHCO₃, 2.5 mmol/l CaCl₂, 0.5 mmol/l EDTA, 5.9 mmol/l KCl, and 1.2 mmol/l MgCl₂. The perfusate was aerated with 95% O₂ and 5% CO₂ and equilibrated at a pH of 7.3 to 7.4. Two side arms in the perfusion line proximal to the heart inflow cannula allowed for infusion of PMNs and plasma directly into the coronary inflow line. Coronary flow was monitored by a flowmeter (Model T106; Transonic Systems, Inc., Ithaca, NY). Left ventricular pressure and the maximal rate of development of left ventricular developed pressure (+dP/dt max) were monitored by using a pressure transducer (Model SPR-524, 2.5F; Millar Instruments, Inc., Houston, TX) that was positioned in the left ventricular cavity. Coronary flow, left ventricular pressure, and +dP/dt max were recorded by using a MacLab data acquisition system (ADI Diagnostics, Castle Hill, Australia) in conjunction with a Power Macintosh 7600 computer (Apple Computers, Cupertino, CA).

Figure 1 illustrates a schematic diagram of the protocol for I/R in the isolated perfused rat heart. Left ventricular developed pressure (LVDP), +dP/dt max, and coronary flow were measured every 5 min for 15 min to equilibrate the hearts and obtain a baseline measurement. LVDP was defined as left ventricular end-systolic pressure minus left ventricular end-diastolic pressure.

After 15 min of equilibration, the flow of Krebs’ buffer was reduced to zero to induce global ischemia for 20 min. At reperfusion, hearts were infused at a rate of 1 ml/min for 5 min with 200 × 10⁶ PMNs resuspended in 5 ml of Krebs’ buffer plus 5 ml of plasma. In some experiments, wortmannin (Biomol, Inc., Plymouth Meeting, PA) was added to plasma at a final concentration of 10, 20, or 40 nM. Sham I/R hearts were not perfused with PMNs and received only plasma. Previous studies showed that sham I/R hearts given PMNs exhibited no changes from initial control values (Lefer et al., 1999). Data were recorded every 5 min for the first 30 min of reperfusion, and at the 45-min time point. After each experiment, hearts were placed in 4% paraformaldehyde and stored at 4°C for subsequent histological analysis.

Isolation of PMNs and Plasma. Sprague-Dawley rats (350 to 400 g), used as PMN donors, were anesthetized with ethyl ether and were given a 14-ml i.p. injection of 0.5% glycogen (Sigma Chemical Co., St. Louis, MO) dissolved in PBS. The rats were reanesthetized with ethyl ether 16 to 18 h later, and the neutrophils were harvested by peritoneal lavage in 30 ml of 0.9% NaCl, as previously described (Lefer et al., 1997). The peritoneal lavage fluid was centrifuged at 250 g for 20 min at 4°C. The PMNs were then washed in 15 ml of PBS and centrifuged at 250g for 10 min at 4°C. Thereafter, the PMNs were resuspended in 2.5 ml of PBS, and a total of 10 samples was pooled before use for cardiac perfusion experiments. The neutrophil preparations were >90% pure and >95% viable, according to microscopic analysis and exclusion of 0.3% trypan blue, respectively.

The plasma used for infusion with the PMNs was isolated from a single rat in each cardiac perfusion experiment to more closely simulate the conditions present in vivo. Blood was collected from the vena cava in citrate phosphate buffer having the following composi-
tion (w/v): 0.3% citric acid, 2.63% trisodium citrate dihydrate, 0.193% sodium phosphate monobasic anhydrous, and 2.32% glucose anhydrous (Sigma), before isolating the heart. The blood was centrifuged at 10,000g for 10 min. Thereafter, the plasma was decanted and later infused into I/R hearts.

**Determination of PMN Infiltration into Cardiac Tissue.** Three rat hearts from each of the eight experimental groups were used for histological analysis. Ten areas of each rat heart were counted for PMN infiltration. Hearts were dehydrated in graded ice-cold acetone washes. The sections were prepared, as previously described (Lefer et al., 1999), and the number of infiltrated PMNs was counted by light microscopy. These results are expressed as infiltrated PMNs per square millimeter of cardiac tissue area.

**PMN Adherence to Superior Mesenteric Artery Endothelium.** Rat PMNs were isolated as reported above. Segments of the superior mesenteric artery (SMA) were removed from control rats, sectioned into 2- to 3-mm rings, opened, and placed into wells containing 2 ml of Krebs-Henseleit buffer. The SMA tissue was challenged with 2 U/ml thrombin to induce P-selectin endothelial surface expression, and then coincubated with fluorescent dye-labeled PMNs (2 × 10⁶ cells), as previously described (Ma et al., 1993). In some SMA segments, wortmannin (10–40 nM) was added to the Krebs-Henseleit buffer before the addition of 2 × 10⁶ PMNs. The number of adherent PMNs was counted by epifluorescence microscopy. Five different fields of each endothelial surface were counted from each vascular segment, and the results are expressed as adherent PMNs per square millimeter of endothelium. An organic NO donor, 4-hydroxymethyl-furazan-3-carboxylic acid-2-oxide, was used as a positive antiadhesive control (Guo et al., 1995).

**Measurement of Superoxide Radical Release from Rat PMNs.** Wortmannin has been previously reported to inhibit human neutrophil superoxide production (Arcaro and Wymann, 1993; Sue et al., 1997). We examined whether wortmannin exerts the same properties. Rat PMNs were isolated as previously described (Guo et al., 1995). We determined the effect of wortmannin on superoxide release from PMNs in response to fMLP stimulation.

**Determination of PMN Infiltration into Cardiac Tissue.** Three rat hearts from each of the eight experimental groups were used for histological analysis. Ten areas of each rat heart were counted for PMN infiltration. Hearts were dehydrated in graded ice-cold acetone washes. The sections were prepared, as previously described (Lefer et al., 1999), and the number of infiltrated PMNs was counted by light microscopy. These results are expressed as infiltrated PMNs per square millimeter of cardiac tissue area.

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**Statistical Analysis.** All data in the text and figures are presented as mean ± S.E. Data on coronary flow, LVDP, and +dP/dt max were analyzed by ANOVA by using post hoc analysis with the Bonferroni/Dunn test. Student’s t test was used to compare final coronary flow, LVDP, and +dP/dt max values between two groups. Probability values of <.05 were considered to be statistically significant.

**Results**

To determine whether wortmannin exerted direct effects on cardiac contractile function, we perfused nonischemic rat hearts with wortmannin at 20 or 40 nM. Perfusion of wortmannin-treated control nonischemic hearts without PMNs did not result in any change in coronary flow, LVDP, or +dP/dt max after 20 min of perfusion. However, hearts from both of these groups recovered to near control values at 45 min of perfusion. In contrast, I/R hearts perfused with PMNs exhibited a 55 to 60% reduction in LVDP at 15 min after reperfusion that was sustained at 45 min of reperfusion, and was significantly lower than the I/R hearts perfused with PMNs given 40 nM wortmannin. However, hearts from both of these groups recovered to near control values at 45 min of perfusion. In contrast, I/R hearts perfused with PMNs given 40 nM wortmannin did not result in any sustained cardiac dysfunction in this model of I/R.

**Time course of cardiac contractile dysfunction (Fig. 2)** indicates a transient decline in LVDP of 30% at 15 min of reperfusion in I/R hearts perfused without PMNs and in I/R hearts perfused with PMNs given 40 nM wortmannin. However, hearts from both of these groups recovered to near control values at 45 min of perfusion. In contrast, I/R hearts perfused with PMNs given 40 nM wortmannin exhibited a 55 to 60% reduction in LVDP at 15 min after reperfusion that was sustained at 45 min of reperfusion, and was significantly lower than the I/R
and I/R + PMN + wortmannin (40 nM) groups at that time (Fig. 2). Moreover, all three wortmannin-treated I/R + PMN groups significantly diminished the postreperfusion cardiac contractile dysfunction, resulting in a significant improvement in final LVDP and +dP/dt max compared with the I/R + PMN group ($P < .01, 10,$ and $20$ nM; $P < .001, 40$ nM) (Figs. 3 and 4).

The I/R + PMN group developed a significant reduction in LVDP, which decreased $44 \pm 5\%$ from initial values ($P < .001$) (Fig. 3), and +dP/dt max, which decreased $53 \pm 6\%$ from initial values at 45 min postreperfusion ($P < .001$) (Fig. 4). The reductions in cardiac contractile performance in the 10, 20, and 40 nM wortmannin + I/R + PMN groups were significantly attenuated compared with the reductions in cardiac contractile performance in the untreated I/R + PMN group (Figs. 3 and 4). Thus, at 45 min postreperfusion, LVDP decreased only $23 \pm 5$, $19 \pm 5$, and $12 \pm 2\%$ from initial values in the 10, 20, and 40 nM wortmannin + I/R + PMN groups, respectively. Similarly, at 45 min postreperfusion, +dP/dt max decreased only $33 \pm 6$, $27 \pm 5$, and $18 \pm 5\%$ from initial values in the 10, 20, and 40 nM wortmannin-treated I/R + PMN groups, respectively. These values are significantly different from the corresponding values in the untreated I/R + PMN group. Additionally, the cardioprotective effect was progressively enhanced as the wortmannin concentration was increased from 10 to 40 nM.
The significant deficit in cardiac performance observed in this study can be associated with the presence of large numbers of PMNs, which infiltrated into the myocardium within 45 min of reperfusion. In this regard, infiltrated PMNs increased from less than 20 PMNs/mm² to more than 200 PMNs/mm² at this time. However, wortmannin, resulted in a 55 ± 6, 69 ± 6, and 73 ± 17% attenuation of PMN infiltration into postreperfused cardiac tissue at 10, 20, and 40 nM wortmannin-treated I/R PMN groups, respectively, compared with the untreated I/R PMN groups (P < .001) (Fig. 5). This inhibition of neutrophil infiltration is viewed as a key component of the cardioprotective effect of wortmannin.

One of the possible mechanisms of the cardioprotective effect of wortmannin is inhibition of superoxide release from infiltrated PMNs. Consistent with this hypothesis, wortmannin significantly reduced superoxide release from suspensions of fMLP-stimulated rat PMNs at 10 and 40 nM wortmannin (P < .001) (Fig. 6).

Another component of the cardioprotective effect of wortmannin may involve inhibition of PMN adherence to the vascular endothelium. Supporting this concept, a significant reduction in PMN adherence to the thrombin-stimulated endothelium also was observed in isolated vascular segments by 10, 20, and 40 nM wortmannin (P < .001) (Fig. 7). These values are comparable with the reduced PMN adherence obtained with the NO donor 4-hydroxymethyl-furazan-3-carboxylic acid-2-oxide, a known inhibitor of PMN adherence to the endothelium (P < .001) (Fig. 7).

**Discussion**

The present study demonstrates that wortmannin exerts significant cardioprotective effects against PMN-mediated reperfusion injury in the isolated perfused rat heart. The cardioprotective effects of wortmannin were characterized by a significant restoration of postreperfusion LVDP and +dP/dt.
max compared with untreated I/R + PMN-perfused rat hearts. These effects of wortmannin are most likely due to significantly reducing PMN adherence to the vascular endothelium, thereby leading to a significant reduction in PMN infiltration into post-perfused cardiac tissue (Knall et al., 1997; Mine et al., 1998). This may be accomplished by suppressing superoxide release from PMNs (Knall et al., 1997; Sue et al., 1997; Mine et al., 1998), thereby attenuating PMN adherence to the vascular endothelium (Mine et al., 1998), and thus reducing PMN infiltration into cardiac tissue (Knall et al., 1997). In this regard, previous studies have shown that oxygen free radicals up-regulate endothelial cell adhesion molecules (e.g., P-selectin) on endothelial cells and quench endogenous NO released from the endothelium (Patel et al., 1991). NO has been shown to act as a physiological inhibitor of leukocyte-endothelial cell interaction by suppressing up-regulation of endothelial cell adhesion molecules (Lefer and Lefer, 1996). In addition, wortmannin also would attenuate superoxide radical release from PMNs that have already migrated into the myocardium, and thus diminish the cardiotoxic effect of oxygen-derived free radicals on cardiomyocytes (Buerke et al., 1994). Therefore, substances like wortmannin that reduce superoxide production from PMNs would tend to attenuate expression of endothelial cell adhesion molecules, effectively diminishing transmigration of PMNs into cardiac tissue and subsequent release of superoxide radicals by PMNs at or near cardiomyocytes. However, reactive oxygen species (i.e., superoxide radicals) are not the sole mechanism of PMN-induced cardiac contractile dysfunction. PMNs can be activated by complement factors (i.e., complement 5a) to release proteases (i.e., elastase) and proinflammatory mediators (i.e., leukotriene B₄) that can contribute to cardiac contractile dysfunction in myocardial I/R injury (Hansen, 1995).

We also have shown that wortmannin directly inhibits superoxide release from neutrophil suspensions, consistent with previous studies, showing a decrease in respiratory burst activity by wortmannin over a similar dose range. This effect can significantly retard the cardiodepressant effects of superoxide radicals directly on cardiac myocytes (Arcaro and Wymann, 1993; Okada et al., 1994; Sue et al., 1997). The 110-kDa catalytic subunit of PI3-kinase is the target for the irreversible inhibition by wortmannin (Ui et al., 1995). Inhibitors of PI3-kinase (i.e., wortmannin) have been shown to inhibit superoxide release from PMNs (Arcaro and Wymann, 1993; Okada et al., 1994; Yan and Novak, 1999). PI3-kinase is a heterodimer of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit that is activated in tyrosine kinase receptor (phosphotyrosine residues) and G-protein-coupled receptor pathways (Ui et al., 1995). PI3-kinase catalyzes transfer of the γ-phosphate of ATP to the D-3 position of phosphatidylinositol (PtdIns), PtdIns4P, and PtdIns(4,5)P₂, and the principal product of the kinase reaction is PtdIns(3,4,5)P₃ (Ui et al., 1995). Active PI3-kinase then activates protein kinase C (PKC), and the kinase in turn phosphorylates the cytosolic factor p47phox that is required for NADPH oxidase activation (Arcaro and Wymann, 1993; Ui et al., 1995). Fully activated NADPH oxidase generates superoxide release from PMNs.

Recent studies using PI3-kinase-deficient mice have shown that the PI3-kinase γ (PI3Kγ) isoform is the isoform responsible for the superoxide release and neutrophil extravasation in PI3Kγ-/- mice, and is required for PMN accumulation in inflammation (Hirsch et al., 2000; Li et al., 2000). PI3Kγ is associated with G-protein receptor responses, whereas the other PI3-kinase isoforms (α, β, and δ) are associated with tyrosine kinase-dependent processes (Hirsch et al., 2000).

Interestingly, PMNs obtained from phospholipase C (PLC) β2 and β3 knockout mice exhibited reduced superoxide rad-
ical release (Li et al., 2000). Li et al. (2000) suggested that both PLC/β3 and PI3K are involved with superoxide radical release. PLC and PI3-kinase are simultaneously activated in response to G-protein receptor-mediated chemoattractants (i.e., fMLP), and both of these signal transduction pathways ultimately converge at the PKC stimulation step (Li et al., 2000). PLC acts via the second messenger diacylglycerol and PI3-kinase through its product PtdIns(3,4,5)P3. PKC phosphorylation of the cyclic factor p47phox is an obligatory step in the activation of NADPH oxidase (Ui et al., 1995). The NADPH oxidase activation is required for the production of superoxide free radicals from PMNs (Ui et al., 1995). Previous studies with phorbol esters (e.g., phorbol-12-myristate-13-ace-tate) that directly activate PKC to elicit superoxide production from PMNs were not inhibited by wortmannin (Arcaro and Wymann, 1993). This is most likely due to PKC activation being downstream of PI3K activation in the signal transduction cascade (Ui et al., 1995).

Wortmannin has not been studied in the PMN-perfused heart subjected to IR. However, previous studies have used wortmannin in myocardial I/R without PMNs (Cittadini et al., 1998; Egert et al., 1999; Otani et al., 2000), and have shown that wortmannin can abolish the positive inotropic effect elicited by insulin-like growth factor-1 (Cittadini et al., 1998; Otani et al., 2000). Moreover, wortmannin also inhibited the myocardial glucose transporter in response to insulin, but not to myocardial ischemia (Egert et al., 1999). These studies suggest that PI3K isoforms (i.e., α, β, and δ) involved with these responses are associated with tyrosine kinase receptor signal transduction, and not the PI3K isoform associated with PMN superoxide release and chemotaxis (Knall et al., 1997). Previous investigations on the effects of wortmannin used isolated PMNs to demonstrate inhibition of PMN superoxide release (Sue et al., 1997), adhesion to endothelial cells (Mine et al., 1998) and chemotaxis (Knall et al., 1997). The results from these studies are consistent with the effects of wortmannin observed in the present study, showing reduced PMN infiltration into I/R cardiac tissue.

In summary, our results are the first to show a cardioprotective effect of wortmannin on PMN-induced myocardial I/R injury in the isolated perfused rat heart. These cardioprotective effects appear to be related to inhibition of PMN superoxide release and PMN adherence to the vascular endothelium, resulting in fewer PMNs infiltrating into cardiac tissue. These effects would result in less superoxide radical release at the level of the cardiomyocytes, thus leading to diminished cardiodepressant effects of PMNs.

References


