Cardioprotective effects of ingliforib, a novel glycogen phosphorylase inhibitor

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Submitted 10 July 2003; accepted in final form 6 November 2003

Tracey, W. Ross, Judith L. Treadway, William P. Magee, Jill C. Sutt, R. Kirk McPherson, Carolyn B. Levy, Donald E. Wilder, Li J. Yu, Yue Chen, Ravi M. Shanker, Alison K. Mutcher, Andrew H. Smith, David M. Flynn, and Delvin R. Knight. Cardioprotective effects of ingliforib, a novel glycogen phosphorylase inhibitor. Am J Physiol Heart Circ Physiol 286: H1177–H1184, 2004. First published November 13, 2003; 10.1152/ajpheart.00652.2003.—Interventions such as glycogen depletion, which limit myocardial anaerobic glycolysis and the associated proton production, can reduce myocardial ischemic injury; thus it follows that inhibition of glycogenolysis should also be cardioprotective. Therefore, we examined whether the novel glycogen phosphorylase inhibitor 5-Chloro-N-{[(3S,2R)-3-[(3R,4S)-3,4-dihydroxy-1-pyrrolidinyl)]-2-hydroxy-3-oxo-1-[(phenylmethyl)propyl]-1H-indole-2-carboxamide (ingliforib; CP-368,296) could reduce infarct size in both in vitro and in vivo rabbit models of ischemia-reperfusion injury (30 min of regional ischemia, followed by 120 min of reperfusion). In Langendorff-perfused hearts, constant perfusion of ingliforib started 30 min before regional ischemia and elicited a concentration-dependent reduction in infarct size; infarct size was reduced by 69% with 10 μM ingliforib. No significant drug-induced changes were observed in either cardiac function (heart rate, left ventricular developed pressure) or coronary flow. In open-chest anesthetized rabbits, a dose of ingliforib (15 mg/kg loading dose; 23 mg/kg·h1·infusion) selected to achieve a free plasma concentration equivalent to an estimated ECS0 in the isolated hearts (1.2 μM, 0.55 μg/ml) significantly reduced infarct size by 52%, and reduced plasma glucose and lactate concentrations. Furthermore, myocardial glycogen phosphorylase a and total glycogen phosphorylase activity were reduced by 65% and 40%, respectively, and glycogen stores were preserved in ingliforib-treated hearts. No significant change was observed in mean arterial pressure or rate-pressure product in the ingliforib group, although heart rate was modestly decreased postischemia. In conclusion, glycogen phosphorylase inhibition with ingliforib markedly reduces myocardial ischemic injury in vitro and in vivo; this may represent a viable approach for both achieving clinical cardioprotection and treating diabetic patients at increased risk of cardiovascular disease.

ischemia; reperfusion; heart; infarct; rabbit

Although the influence of ischemia and reperfusion on cardiac metabolism has been extensively investigated (see Refs. 5, 16, and 24 for reviews), less well defined are the ways in which pharmacological manipulation of cardiac metabolism may be cardioprotective. Under ischemic conditions, myocardial oxidative metabolism is suppressed and glycolysis becomes an important source of ATP generation (32). The increased glycolytic rate in the face of impaired glucose oxidation leads to uncoupling of the two pathways and a buildup of lactate and H+ (4, 16), a process which may continue during reperfusion (22). This accumulation of protons leads to downstream activation of pathways (Na+/H+ exchanger, Na+/Ca2+ exchanger) that result in Ca2+ overload, impaired contractile function, and/or cell death. Therefore, the approaches that are able to improve glycolytic/oxidative coupling by reducing the glycolytic rate could be expected to be cardioprotective.

One possible approach would be to reduce myocardial glycolysis and thus restrict a source of substrate for glycolysis. Several studies (3, 7, 28, 30, 42, 43) examining the mechanistic basis of ischemic preconditioning have demonstrated in preconditioned hearts that myocardial glycogen stores are depleted, accompanied by attenuated glycolysis and glycolysis, and reduced accumulations of lactate and protons. Moreover, the loss of myocardial protection in preconditioned hearts correlates with the time course of glucose recovery (43). Experimental manipulations designed to deplete myocardial glycogen before ischemia-reperfusion also have been shown to be cardioprotective (1, 19, 31). Nevertheless, the ability of glycogen to modulate ischemia-reperfusion injury is controversial in that other studies (10, 15, 20, 37) have failed to show either a link between glycogen depletion and ischemic preconditioning, or a cardioprotective benefit of reducing glycogen stores before ischemia and reperfusion.

Given that both glycolysis (30, 42) and conversion of glycogen phosphorylase (GP) to the active (a) form (GPa) (42) are reduced in preconditioned hearts, and both GP activity and glycolysis are increased during ischemia in nonpreconditioned hearts (8), pharmacological inhibition of GP, and thus glycolysis, could be postulated to be cardioprotective. A limitation facing past investigations was the lack of pharmacological tools with which to specifically inhibit GP, although α-1,6-glucosidase glycogen debranching enzyme inhibitors N-hydroxyethyl-1-deoxyribozyme (miglitol) and N-methyl-1-deoxyribozyme (MOR-14) have been reported to reduce both myocardial glycogen breakdown and infarct size (2, 29). Nevertheless, the putative cardioprotective benefit of inhibiting GP has not been formally demonstrated. We recently described a novel class of GP inhibitors (12, 27), which bind at a newly discovered allosteric binding site on the enzyme (34). One of

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these inhibitors is 5-Chloro-N-{(1S,2R)-3-[(3R,4S)-3,4-dihydroxy-1-\(\text{pyrrolidinyl}\)-2-hydroxy-3-\(\text{oxo}\)-1-(\text{phenylmethyl})propyl]-1H-indole-2-carboxamide (ingliflorib; CP-368,296) (13) (Fig. 1), which inhibits the GP isoforms expressed in the myocardium with IC_{50} values of 352 nM (muscle GP) and 150 nM (brain GP), respectively. Thus, to help further clarify the involvement of glycogenolysis in myocardial ischemia-reperfusion injury, we used this novel compound to investigate whether GP inhibition is cardioprotective in both in vitro and in vivo rabbit models of ischemia-reperfusion injury.

**MATERIALS AND METHODS**

This investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

**In vitro Langendorff preparation.** Male New Zealand White rabbits (3 to 4 kg; Covance; Denver, CO) were anesthetized by intravenous administration of pentobarbital sodium (30 mg/kg), followed by intubation and ventilation with 100% O_{2} with the use of a positive pressure ventilator. A left thoracotomy was performed, the heart exposed, and a snare (2-0 silk) was placed loosely around a prominent branch of the left coronary artery. The heart was rapidly removed from the chest, mounted on a Langendorff apparatus, and maintained by perfusion (nonrecirculating) with a modified Krebs solution composed of (in mM) 118.5 NaCl, 4.7 KCl, 1.2 MgSO_{4}, 1.2 KH_{2}PO_{4}, 24.8 NaHCO_{3}, 2.5 CaCl_{2}, and 10 glucose at a constant pressure of 80 mmHg and a temperature of 38.5°C. Perfusion pH was maintained at 7.4 to 7.5 by bubbling with 95% O_{2}-5% CO_{2}. The temperature of the heart was maintained by suspending it in a heated, water-jacketed organ bath. A fluid-filled latex balloon was inserted in the left ventricle and connected by stainless steel tubing to a pressure transducer; the balloon was inflated to provide a systolic pressure of 80–120 mmHg, and a diastolic pressure ≈10 mmHg. Heart rate (HR), left ventricular (LV) systolic and diastolic pressures, and LV developed pressure (LVDP) were recorded using a PO-NE-MAH Data Acquisition and Archive System (Gould Instrument Systems; Valley View, OH). Total coronary flow (CF) rate was determined using an in-line flow probe (Transonic Systems; Ithaca, NY); CF was normalized for heart weight. Each heart was allowed to equilibrate for 30 min; if stable LV pressures within the parameters outlined above were not observed, the heart was discarded. Pacing was not used unless the heart rate fell <180 beats/min before the 30-min period of regional ischemia; in this case, the heart was paced at 200 beats/min, which was the average spontaneous rate observed.

**Langendorff experimental protocols.** After a 30-min equilibration period, a constant perfusion with ingliflorib was initiated, and continued for the duration of the experiment. Thirty minutes after drug perfusion was started, a 30-min period of regional ischemia was produced by tightening the snare around the branch of the coronary artery. At the end of the ischemic period, the snare was released, and the heart reperfused for an additional 120 min. In control hearts, the heart was reperfused for an additional 120 min. Myocardial ischemia was confirmed by reactive hyperemia and rapid decline of the ST elevation. At the end of either the ischemic period or reperfusion period, each rabbit was euthanized with an intravenous overdose of pentobarbital sodium (100 mg/kg). The heart was quickly excised and prepared for measurement of MAP, activity and glycogen content, or mounted on a Langendorff apparatus and perfused with physiological saline at 38.5°C for subsequent determination of infarct size.

**Determination of infarct size.** After completion of each experiment (in vitro or in vivo) and with the heart suspended and perfused on the Langendorff apparatus, the coronary artery snare was retightened, and then 1% triphenyl tetrazolium chloride in phosphate-buffered saline (1–10 μm) was perfused through the heart to delineate the area-at-risk (AAR; nonlabeled) in the LV for infarct development. The heart was removed from the Langendorff apparatus, blotted dry, weighed, wrapped in aluminum foil, and stored overnight at −20°C. Frozen hearts were sliced into 2-mm transverse sections and incubated with 1% triphenyl tetrazolium chloride in phosphate-buffered saline for 20 min at 37°C to delineate noninfarcted (stained) from infarcted (nonstained) LV tissue. The infarct area (IA) and the AAR were calculated for each slice of LV using video-captured images and image analysis software (model ETC3000, Engineering Technology Center; Mystic, CT), followed by adding the values for each tissue slice to obtain the total IA and total AAR for each heart. To normalize the infarct area for differences in the AAR between hearts, the infarct size was expressed as the ratio of IA versus AAR (%IA/AAR).

**Determination of drug concentrations in plasma and protein binding.** Quantitation of ingliflorib was accomplished with the use of a liquid chromatography/tandem mass spectrometry (LC/MS/MS) instrument (model API3000, PE-Sciex; Toronto, Canada). An aliquot (10 μl) of plasma or tissue homogenate (0.2 g/ml in 10 mM sodium phosphate buffer at pH 7.4) was precipitated using 200 μl of methanol-acetonitrile (1:1). After centrifugation, an aliquot (40 μl) of supernatant was diluted with 200 μl of methanol-acetonitrile (1:1), and the diluted sample (5 μl) was injected onto a Phenomenex 40 × 2 mm 5 μm C18 column maintained at 37°C with a run time of ~3 min. The analyte was eluted at 0.5 ml/min flow rate with a linear gradient program consisting of methanol (pump A, 5–95% ramping) and 10 mM ammonium acetate (pump B, 95–5% ramping) produced by two Shimadzu LC-10A VP binary pumps and a 10-μl static mixer. The column effluent was analyzed using a TurboIonSpray source at 500°C of a PE-Sciex API-3000 triple quadrupole mass spectrometer. Ingliflorib was detected at m/z 456.2 → 193.0 at a retention time of 1.65 min. The calibration curve was prepared by...
addition of authentic standard (ingliforib) to the control plasma or control tissue homogenate, at concentrations of 0.05 to 50 μg/ml for the plasma and 0.1 to 50 μg/ml for the tissue (6 to 7 concentrations per standard curve). The standards were processed as the unknowns described above. The standard curve was obtained by fitting linear least-squares regression analysis from the peak area of ingliforib with 1/(concentration)² weighting. The acceptance criterion for the analysis was that all standards used in the curve were ±20% absolute deviation from the normal value. The absolute tissue-to-plasma concentration ratio was found to be ~1.5 in heart and ~2.7 in liver after a 2-h infusion (at steady state).

Plasma protein binding was determined by a 96-well equilibrium dialysis apparatus. Spectro-pro number 2 membranes with molecular weight cutoff of 12-14 kDa were used for the study and were conditioned for 15 min in deionized water, 15 min in 30% ethanol, and 30 min in sodium phosphate buffer (pH 7.4; 100 mM). Fresh rabbit plasma was obtained from control animals on the day of the study. Plasma samples were spiked with ingliforib to achieve a concentration of 1 μg/ml; 150-μl aliquots (n = 6) were loaded into the 96-well equilibrium dialysis apparatus and dialyzed against 150 μl of sodium phosphate buffer. Equilibrium was achieved by incubating the 96-well equilibrium dialysis apparatus in a 37°C shaking water bath at 155 rpm for 5 h. At the end of the dialysis period, 10 μl of the dialyzed plasma and 90 μl of the buffer were transferred to HPLC vials containing 100 μl of methanol-acetonitrile (1:1). Control buffer (90 μl) was added to the vial containing the dialyzed plasma sample, and 10 μl of control plasma was added to the vial containing the buffer sample. The vials were vortexed and centrifuged, and the supernatant was assayed by the LC/MS/MS assay described above. The plasma unbound fraction (f_u) was estimated by the ratio of drug concentration in the buffer sample to the drug concentration in the plasma sample. (The mean f_u for ingliforib was 0.036 ± 0.002 in rabbit plasma).

**Determination of plasma glucose and lactate concentrations.** Blood samples were collected in heparinized tubes, followed by centrifugation and collection of the plasma. Plasma glucose and lactate concentrations were determined with the use of a Roche/Hitachi 912 Clinical Autoanalyzer (Roche Diagnostics, Indianapolis, IN) using the Glucose HK and Lactate reagent systems (Roche Diagnostics), respectively.

**Measurement of myocardial GP activity.** Hearts were rapidly removed from the animals at the end of the 30-min ischemic period and perfused with ice-cold saline. The ischemic myocardium was identified as the region not cleared of blood by the saline perfusion and was dissected free of the remainder of the heart. In hearts in which infarct size was determined, the right ventricular free wall was used for determining GP activity. Myocardial samples were frozen in liquid nitrogen and stored at ~80°C until analysis. Heart samples (75 mg) were homogenized at a 1:39 dilution in 50 mM MES, 100 mM potassium fluoride, 5 mM EDTA, and 0.4% β-mercaptoethanol, pH 6.1, with the use of a Vertis Handishear at 30,000 rpm for 15 s. Samples were then centrifuged at 3,000 g for 5 min, and the supernatant transferred for analysis of GP activity by modification of the method of Gilboe et al. (9). In brief, a 60-μl reagent mix composed of 50 mM MES, 75 mM KF, 0.8% glycerogen, 50 mM glucose-1-phosphate, 45 nCi [14C]glucose-1-phosphate (NEC390, Perkin Elmer), and ± 3.3 nM AMP, pH 6.1, was pipetted into 12 × 75 glass tubes. To initiate the reaction, a 30-μl sample was added to the tubes in duplicate, and the reaction allowed to proceed at 37°C for 30 min. The reaction was terminated by the removal of a 75-μl aliquot and by spotting onto a 15 × 15 mm Whatman 31ET CHR filter paper. Filters were washed three times with 60% ethanol, dried with acetone, and placed in 7-mI scintillation vials with 5.5 ml of scintillation fluid (Beckman Coulter Ready Safe), and counted on a liquid scintillation counter (model 1409, Wallac). The results are expressed in units of disintegrations per minute per milligram tissue, and analyzed in duplicate. Cardiac GPa activity is defined as the measured activity in the absence of AMP (−AMP); total GP activity (GPa + GPb) is defined as the measured activity in the presence of AMP (+AMP).

**Measurement of myocardial glycogen content.** At the end of the 30-min period of regional ischemia, hearts were removed from the animals and rapidly perfused with ice-cold saline. The ischemic myocardium was identified as the region not cleared of blood by the saline perfusion and was dissected free of the remainder of the heart. Myocardial samples were frozen in liquid nitrogen, and stored at ~80°C until analysis. Approximately 25 mg of frozen tissue were added to 16 × 100 mm glass test tubes, followed by the addition of 1.5 ml of 30% KOH. The tubes were heated in a 60°C oven for 30 min and repeatedly agitated. Two milliliters of 100% ethanol and 250 μl of saturated sodium sulfate were added to each sample. The tubes were heated for 3 min at 90°C, and then placed on ice for 15 min. Samples were centrifuged at 4°C, 3,200 g for 5 min. After aspiration of the supernatants, the pellets were dried for 60 min in a 60°C oven. The pellets were then hydrolyzed in 1 ml of 5N HCl for 1 h in a 60°C oven. Samples were cooled at room temperature and neutralized with 1 ml of 5N NaOH and 3 ml of deionized water. For each sample, 2 ml of anthrone reagent [200 mg of anthrone (Sigma) in 100 ml H2SO4] were added to a 16 × 100 mm test tube, followed by the addition of 1 ml of neutralized heart hydrolysate or 1 ml of glucose standard. Tubes were vortexed and heated at 90°C for 15 min; samples were then immediately cooled at 4°C. Two hundred microliters were transferred in duplicate to a 96-well plate, which was read at 620 nm in a SpectroMax Plus microplate reader (Molecular Devices; Sunnyvale, CA).

**Data expression and analysis.** Data are expressed as the means ± SE. Between group comparisons of in vitro and in vivo AAR expressed as a percentage of LV areas (%AAR/LV) were compared using ANOVA. Temporal comparisons of in vivo hemodynamic parameters, plasma glucose concentrations, and plasma lactate concentrations between ingliforib and vehicle control were performed using ANOVA with repeated measures. In vitro hemodynamic, glycogen content, and GP activity comparisons were performed by t-test, whereas in vitro and in vivo %IA/AAR values were compared using a Mann-Whitney test; a Bonferroni correction was applied to multiple comparisons. A P value of <0.05 was considered statistically significant.

**Drugs and drug preparation.** The synthesis of (ingliforib; CP-368,296) has been reported (13) and was performed at Pfizer Global Research and Development (Groton, CT). Drug administered to the isolated hearts was dissolved in DMSO and diluted in buffer; the final DMSO concentration was <0.1%, which had no effect on infarct size (39). For the in vivo studies, ingliforib was dissolved in 25% sulfobutylether 7-β-cyclodextrin sodium (Captisol, Cydex; Overland Park, KS) in 0.01 M phosphate-buffered saline at a concentration of 13 mg/ml.
**RESULTS**

In the isolated rabbit hearts, baseline HR, CF, and LVDP values for each of the treatment groups were similar before the regional ischemia and are shown in Table 1. LVDP and CF were significantly (P < 0.05) reduced in all groups by occlusion of the coronary artery, confirming that ischemia was achieved in all groups. In anesthetized rabbits, baseline HR, MAP, and RPP were similar between vehicle control and ingliforib-treated groups (Fig. 2). Administration of ingliforib did not significantly affect HR, LVDP, or CF in the isolated hearts (Table 1), nor did this compound affect MAP or RPP in vivo (Fig. 2, B and C). A modest reduction in HR of the ingliforib group versus the vehicle control group (P < 0.05) was observed during the reperfusion period in the in vivo studies (Fig. 2A).

Ingliforib elicited a concentration-dependent reduction in infarct size in the isolated rabbit hearts (Fig. 3). The maximum reduction in infarct size achieved with 10 μM ingliforib was 69% (control, 52 ± 2% IA/AAR; 10 μM ingliforib, 16 ± 2% IA/AAR, P < 0.05). %AAR/LV for the ingliforib treatment groups did not differ significantly (P ≥ 0.05) from that of the control group (33 ± 2%). In anesthetized rabbits, a dose of ingliforib was selected to achieve free drug plasma concentrations comparable to an EC50 concentration (1.2 μM, 0.55 μg/ml) estimated from the isolated heart experiments. This dose of ingliforib (15 mg/kg loading dose; 23 mg·kg⁻¹·h⁻¹ infusion) provided a plasma concentration of 21.0 ± 1.4 μg/ml just before the regional ischemia; ingliforib is 96.5% protein bound, yielding a free drug plasma concentration of 0.7 μg/ml (1.5 μM). At this dose, infarct size was significantly reduced by 52% in vivo (Fig. 4) (vehicle control: 65 ± 3% IA/AAR; ingliforib: 31 ± 4% IA/AAR, P < 0.05); the %AAR/LV did not differ (P > 0.05) between these groups (control: 41 ± 5%; ingliforib: 42 ± 4%).

GP activity was significantly (P < 0.05) inhibited in the myocardium from the ingliforib-treated animals (Fig. 5). At the end of the 30-min period of regional ischemia, GPa and total GP activity were reduced by 65% and 40%, respectively, in the ischemic myocardium, and 41% and 33%, respectively, in the nonischemic myocardium (Fig. 5). In addition, the ingliforib-dependent GPa inhibition was significantly (P < 0.05) greater in the ischemic versus nonischemic myocardium. GPa and total GP activity were similar in the ischemic and nonischemic myocardium from the vehicle-treated animals (Fig. 5). Inhibition of GP activity by ingliforib was also verified in hearts in which infarct size was determined by measuring GP activity in the right ventricle; GPa and total GP activity were reduced by 83% (vehicle: 4,164 ± 699 dpm/mg tissue, ingliforib: 666 ± 115 dpm/mg tissue; n = 8) and 63% (vehicle: 7,044 ± 1,003 dpm/mg tissue, ingliforib: 2,622 ± 247; n = 8), respectively, at the end of the reperfusion period.

To establish inhibition of glycogenolysis by ingliforib, glycogen content in the ischemic and nonischemic myocardium from vehicle- and ingliforib-treated anesthetized rabbits was measured at the end of the 30-min period of regional ischemia. Myocardial glycogen stores were significantly (P < 0.05) reduced in the ischemic versus nonischemic myocardium, whereas ingliforib treatment significantly (P < 0.05) preserved glycogen content in the ischemic myocardium (Fig. 6).

Systemic GP inhibition by the cardioprotective dose of ingliforib was assessed by measuring plasma glucose and lactate concentrations. Baseline plasma glucose and lactate concentrations were comparable in vehicle and ingliforib-treated groups (Fig. 7). In vehicle control animals, a rise in plasma glucose and lactate concentrations were observed during the ischemic period, which peaked at the end of the ischemia and remained elevated during the subsequent reperfusion. Ingliforib significantly (P < 0.05) blunted the rise in both glucose and lactate plasma concentrations (Fig. 7).

**DISCUSSION**

Under the anaerobic conditions of myocardial ischemia, the heart relies primarily on glycolysis for ATP generation (32). Myocardial glycogen and glycogenolysis are important sources of glycolytic substrate, particularly when coronary flow is limited (5) and exogenous glucose delivery to the heart is reduced. However, because oxidation is impaired during ischemia, glycogenesis and oxidation become uncoupled, leading to a buildup of lactate and H⁺ (4, 16). This deleterious process can continue during reperfusion because glycolysis and fatty acid oxidation (on which the heart primarily depends for its energy demands) recover quickly and may exceed preischemic rates (21, 25, 36). Consequently, glucose oxidation remains markedly depressed, glycolytic/oxidative uncoupling continues, and the myocardial lactate/proton load persists (24, 25, 35). When we consider these observations, it follows that inhibition of glycogenolysis would be cardioprotective due to a reduction in glycolytic substrate and improved glycolytic/oxidative coupling; indeed, studies (30, 42) in preconditioned hearts have demonstrated glycogenolysis is significantly attenuated. Weiss et al. (42) established this decrease in glycogenolysis (likely due to the reduced conversion of GP to the “a” or “active” form during early ischemia) resulted in a diminished glycolytic rate and decreased accumulation of lactate and H⁺. Conversely, GP activity and glycogenolysis have been reported to increase markedly during global low-flow ischemia in non preconditioned hearts (8).

**Table 1. Cardiac function and coronary flow data from isolated rabbit hearts**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Preischemia</th>
<th>End Ischemia</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR, beats/min</td>
<td>CF, ml·min⁻¹·g⁻¹</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>213 ± 10</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>Ingliforib (0.1 μM)</td>
<td>6</td>
<td>208 ± 11</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>Ingliforib (1 μM)</td>
<td>6</td>
<td>207 ± 7</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>Ingliforib (10 μM)</td>
<td>10</td>
<td>203 ± 3</td>
<td>7.0 ± 0.3</td>
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Values are means ± SE; n, no. of rabbit hearts. HR, heart rate; CF, total coronary flow; LVDP, left ventricular developed pressure. *P < 0.05 vs. preocclusion values.
To formally establish that pharmacological inhibition of GP is cardioprotective, we used a novel GP inhibitor, ingliforib, in well-established models of myocardial ischemia-reperfusion injury. Ingliforib inhibits the myocardial GP isoforms (muscle and brain) with IC₅₀s of 352 and 150 nM, respectively, and is also a potent inhibitor of the liver isoform (IC₅₀ of 52 nM). By using this compound, we have demonstrated for the first time that inhibition of myocardial GP provides significant protection from myocardial ischemia-reperfusion injury. The cardioprotection afforded by ingliforib in the isolated rabbit heart was concentration dependent; 10 μM ingliforib reduced infarct size by 69%, which is similar to the efficacy of other cardioprotect...
tive agents we have characterized in this model (e.g., adenosine A\_3 receptor agonists, Na\(^+\)/H\(^+\) exchanger inhibitors, Na\(^+\)/Ca\(^2+\) exchanger inhibitors, aldose reductase inhibitors) (18, 26, 40, 41). In vivo studies, which were designed to target a free plasma concentration equivalent to the EC\(_{50}\) we estimated from the isolated heart studies, resulted in a 52% reduction in infarct size. In addition, GP inhibition was confirmed in vivo, both within the heart and systemically. GP activity (total and GPa) was significantly blunted by ingliforib in the ischemic and nonischemic myocardium, and glycogen stores preserved in the ischemic myocardium. Systemically, plasma glucose and lactate concentrations were significantly lowered by ingliforib treatment. However, the in vivo cardioprotective efficacy of ingliforib was independent of systemic GP inhibition because ingliforib reduced infarct size in vitro, and equivalent drug exposure in vitro and in vivo produced similar reductions in infarct size. It was noteworthy that neither in the isolated heart, nor in vivo, were any significant unwanted cardiovascular effects observed, i.e., changes in cardiac function, coronary flow, or mean arterial blood pressure. In vivo, heart rate was minimally reduced in the ingliforib-treated group; whereas this could be viewed as a trend toward reducing myocardial oxygen consumption, a significant drop in RPP was not observed. Our results show that partial (65–83%) inhibition of cardiac GP was associated with reduced infarct size in the absence of other untoward effects on cardiac function. Whether complete inhibition of cardiac GP in the ischemic myocardium would produce a similar profile, or would lead to untoward effects due to energy substrate deprivation, remains to be determined.

Our data support earlier studies in which α-1,6-glucosidase glycogen debranching enzyme inhibitors (miglitol, MOR-14) preserved myocardial glycogen content, attenuated lactate accumulation and reduced infarct size (2, 29). The demonstration that ingliforib has similar effects on myocardial glycogen content and infarct size further underscores the significance of inhibiting glycogenolysis for ameliorating myocardial ischemia-reperfusion injury, while validating GP as a cardioprotec-
tive molecular target. Moreover, the in vivo efficacy of ingliforib and lack of adverse effects suggests that GP inhibition may be a viable therapeutic approach for achieving clinical cardioprotection. As a pharmacological tool, ingliforib should facilitate further study of the role of GP in the physiology/pathophysiology of the heart and other organs.

Although these studies focused on the response of normal hearts to ischemia-reperfusion injury, GP inhibitors are being investigated for the treatment of diabetes (ingliforib reduced plasma glucose and lactate in our normal rats, and reduces plasma glucose in diabetic models (D. J. Hoover, E. M. Gibbs, and J. L. Treadway, unpublished observations)). Moreover, diabetic patients have an increased risk for developing cardiovascular complications, including myocardial infarction (11, 14). Although controversial (6, 33), the almost complete reliance of the diabetic heart on fatty acid metabolism and minimal glucose oxidation rate may increase the sensitivity to ischemic injury due to the considerable uncoupling of glycolysis and glucose oxidation (23, 24, 33, 38). Thus one could speculate that a GP inhibitor might not only treat diabetes per se, but may also protect the diabetic heart already predisposed to ischemic injury. Future studies to address this possibility should be considered.

In conclusion, we have demonstrated that a novel GP inhibitor, ingliforib, inhibits myocardial GP, preserves glycogen stores, and provides significant cardioprotection from ischemia-reperfusion injury. The benefit resulting from GP inhibition may ultimately be due to a reduction in myocardial glycolysis, an improvement in glycolytic/oxidative coupling, and a reduction in intracellular proton load. Moreover, the cardioprotection is achieved without eliciting undesirable changes in cardiac function or hemodynamics. Thus GP inhibition may represent an attractive target for clinical cardioprotection and for treating diabetic patients at increased risk for cardiovascular complications.

ACKNOWLEDGMENTS

The authors thank Drs. Dennis J. Hoover, E. Michael Gibbs, Shawn C. Black, and Roberto A. Calle for support and input during these studies.

DISCLOSURES

All of the authors are employees of Pfizer and have financial interests in the company.

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