Enhancement of Glutathione Cardioprotection by Ascorbic Acid in Myocardial Reperfusion Injury

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ABSTRACT

The present experiment determined the effects of glutathione and ascorbic acid, the two most important hydrophilic antioxidants, on myocardial ischemia-reperfusion injury and evaluated their relative therapeutic values. Isolated rat hearts were subjected to ischemia (30 min) and reperfusion (120 min) and treated with ascorbic acid, glutathione monoethyl ester (GSHme), or their combination at the onset of reperfusion. Administration of 1 mM GSHme alone, but not 1 mM ascorbic acid alone, significantly attenuated posts ischemic injury (P < 0.05 versus vehicle). Most interestingly, coadministration of ascorbic acid with GSHme markedly enhanced the protective effects of GSHme (P < 0.01 versus vehicle). The protection exerted by the combination of GSHme and ascorbic acid at 1 mM each was significantly greater than that observed with 1 mM GSHme alone (P < 0.05). Moreover, treatment with GSHme alone or GSHme plus ascorbic acid markedly reduced myocardial nitrotyrosine levels, suggesting that these treatments attenuated myocardial peroxynitrite formation. These results demonstrated that 1) GSHme, but not ascorbic acid, exerted protective effects against ischemia-reperfusion injury; and 2) the protective effects of GSHme were further enhanced by coadministration with ascorbic acid, suggesting a synergistic effect between GSHme and ascorbic acid.

Ischemia plays a central role in diseases such as myocardial infarction and stroke. Recent advances in thrombolytic therapy have made it possible to reverse ischemia at an early stage. However, reperfusion with oxygen, considered the mainstay of treatment for ischemia, may actually contribute to further tissue and cell damage inducing a process termed reperfusion injury.

It is well recognized that reperfusion injury occurs through the generation of reactive oxygen species (ROS) and derangements in calcium homeostasis (Maxwell and Lip, 1997). The burst production of ROS during the early phase of reperfusion can cause microvascular damage, endothelial cell dysfunction, and accelerated cell death. Theoretically, exogenous supplementation of antioxidants should restore the antioxidant/oxidant balance, thus reducing oxidative tissue injury and improving cardiac function. However, experiments in which selective antioxidants, such as superoxide dismutase and catalase, have been administered have yielded mixed results (Bolli, 1991). A recent study reported that tissue content of glutathione (GSH) and ascorbic acid is significantly reduced in ischemic reperfused myocardial tissue (Haramaki et al., 1998). In contrast, other antioxidants, such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and α-tocopherol were unchanged unless additional H2O2 was administered during ischemia and reperfusion (Haramaki et al., 1998). These results demonstrated that the tissue hydrophilic antioxidants are the first line of antioxidant defenses during reperfusion, and suggested that exogenous supplementation of GSH and ascorbic acid may exert superior protective effects against reperfusion injury compared with other antioxidants.

Early studies have demonstrated that there is significant overlap in the functions of GSH and ascorbic acid in the destruction of ROS. In newborn rats with GSH depletion [by treatment with L-buthionine-(S,R)-sulfoximine], administration of ascorbic acid prevented tissue injury and death (Martensson et al., 1991). On the other hand, in guinea pigs depleted of ascorbic acid (by giving an ascorbic acid-deficient diet), supplementation of GSH ester significantly delayed the onset of scurvy and attenuated tissue injury in these animals (Martensson et al., 1993). These results indicated that GSH

ABBREVIATIONS: ROS, reactive oxygen species; GSH, glutathione; KH, Kreba-Henseleit; LVEDP, left ventricular developed pressure; LVSP, left ventricular systolic pressure; LVDP, left ventricular developed pressure; LvdP/dtmax, the maximal rate of the first derivation of left ventricular pressure; GSHme, glutathione monoethyl ester; sham MI, sham ischemic-reperfusion heart; VF, ventricular fibrillation; PBS, phosphate-buffered saline; ONOO-, peroxynitrite anion; BSA, bovine serum albumin; TBAR, thiobarbituric acid reactive substance; bpm, beats per minute.
and ascorbic acid can compensate for each other under experimental conditions in which one of them is selectively depleted. However, interactions between these two functionally similar antioxidants and their relative therapeutic values (i.e., whether an optimal protection could be achieved by administering one of them alone) in myocardial ischemia and reperfusion, a pathological condition in which both GSH and ascorbic acid are significantly decreased, has not been elucidated.

Therefore, the purposes of the present study were to 1) compare the effects of exogenously administered GSH and ascorbic acid on reperfusion injury; 2) investigate the interrelationship between GSH and ascorbic acid in their cardioprotective effect against reperfusion injury (i.e., replaceable, additive, or synergistic); and 3) determine the mechanisms by which ascorbic acid and GSH may exert their cardioprotective effects against reperfusion injury in an isolated perfused rat heart model.

Experimental Procedures

Heart Preparation and Measurement of Functional Parameters

Rats were anesthetized (sodium pentobarbital, 50 mg/kg i.p.) and heparinized (sodium heparin, 1000 U/kg i.v.). Five minutes after heparin injection, a midsternal thoracotomy was performed, and the hearts were rapidly excised and immediately placed into ice-cold Krebs-Henseleit (KH) solution consisting of 118 mM NaCl, 4.75 mM KCl, 1.19 mM KH2PO4, 1.19 mM MgSO4·7H2O, 1.8 mM CaCl2·2H2O, 25 mM NaHCO3, and 11 mM glucose. Within 30 s, they were mounted onto a Langendorff heart perfusion apparatus (Radnoti Glass Technology, Monrovia, CA), and perfused with KH solution (37°C, oxygenated with 95% O2–5% CO2, pH 7.35–7.45) at a constant pressure of 60 mm Hg. The coronary flow rate was measured via an in-line flow probe connected to an ultrasonic flowmeter (Transonics Systems, Ithaca, NY).

After the hearts were mounted onto the perfusion apparatus, the left atrium was incised and a Millar micro-tip catheter transducer (SPR-524, 2.5F) inserted into the left ventricle through the mitral orifice. Left ventricular end diastolic pressure (LVEDP) was adjusted to 6 to 8 mm Hg by immersing the heart in a water-jacketed heart chamber filled with 37°C KH buffer solution exposed to room air (Lefer et al., 1999). Traditionally, left ventricular function in isolated hearts is assessed by the pressure obtained by filling the left ventricular cavity with a fluid-filled balloon connected to a pressure transducer. The major problem using this method is that the balloon itself can cause additional myocardial injury, especially during the global ischemic period during which a substantial myocardial contracture occurs. To avoid this problem, a new technique has been developed recently by using a Millar micro-tip catheter transducer (Pabla et al., 1996). Because global ischemia causes a marked myocardial contracture and LVEDP remained at a value greater than 20 mm Hg, the left ventricle cannot be filled with solution in the heart chamber. Therefore, no cardiac output could be observed after reperfusion. Left ventricular pressure and coronary flow rate were continuously monitored and recorded on a computer via a Digi-Med heart performance system (Micro-Med, Louisville, KY). The left ventricular systolic pressure (LVSP), LVEDP, left ventricular developed pressure (LVEDP = LVSP – LVEDP), maximal rate of the first derivative of left ventricular pressure (LVdP/dtmax), heart rate, and mean coronary flow rate were derived by computer algorithms at control (before ischemia), and 10, 30, 60, and 120 min after reperfusion.

Biochemical Determination of Myocardial Injury

At the end of 120-min reperfusion, the heart was removed from the perfusion apparatus and divided into four parts for biochemical assays as described below.

Myocardial Creatine Kinase Content. Myocardial tissue was homogenized in cold 0.25 M sucrose (1:10, w/v) containing 1 mM EDTA and 0.1 mM mercaptoethanol using a PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT). Homogenates were centrifuged at 36,000g for 30 min. The supernatants were decanted and analyzed spectrophotometrically for myocardial creatine kinase activity as reported previously (Ma et al., 1997).

Myocardial GSH Content. Myocardial tissue glutathione concentration was quantified using a glutathione reductase–5,5'-dithiobis-(2-nitrobenzoic acid)-based enzymatic recycling assay. Heart tissue was washed with ice-cold phosphate-buffered saline and homogenized in 5% 5-sulfosalicylic acid on ice. After centrifugation at 10,000g for 5 min, supernatant was collected for the assay. To each well of a 96-well plate, 25 μl of standards or samples, 25 μl of sample buffer (143 mM NaH2PO4, and 6.3 mM EDTA-Na), and 200 μl of working solution (10 mM glutathione reductase–5,5'-dithiobis (2-nitrobenzoic acid) and 2 mM NADPH in sample buffer) were added. The reaction was initiated by adding 50 μl of 7.2 IU of oxidized glutathione-reductase (G-3664; Sigma Chemical, St. Louis, MO) to each well, and the absorbance change at 405 nm was continuously monitored for 2 min at a medium intensity, 30 s. The homogenates were centrifuged for 10 min at 12,500g for 4°C. The supernatants were collected and protein concentrations were determined as described below. A nitrated protein solution was prepared for use as a standard by adding 8 μl (2 μl x 4) of chemically synthesized peroxynitrite anion (ONOO-) (concentration, 100–120 mM) to 3 ml of 0.04% (0.4 mg/ml) BSA in PBS. The amount of nitrotyrosine present in the peroxynitrite-treated BSA solution was measured at 430 nm (εmax = 4400 M−1 cm−1) using a SpectraMax-Plus microplate spectrophotometer ( Molecular Devices) and expressed as nanograms per milliliter. The stock solution of the peroxynitrite-treated BSA was diluted with PBS (final concentration of 0.005 before drug administration, 7.313 ± 0.004 during infusion of ascorbic acid and GSHme at a final targeted concentration of 1 mM each). Sham ischemic-reperfusion hearts (sham MI) were perfused with KH solution for 150 min. Reperfusion-induced ventricular fibrillation (VF) was defined according to the Lambeth Conventions (Walker et al., 1988), and the incidence of ventricular tachyarrhythmias and VF were compared among the groups.

Myocardial Nitrotyrosine Content. Quantification of myocardial tissue nitrotyrosine levels was performed by using a modified enzyme-linked immunosorbent assay procedure (Tanaka et al., 1998; Ronson et al., 1999). In brief, myocardial tissue was homogenized in ice-cold PBS (1/10 w/v) using a PRO 200 homogenizer followed by sonication with a dismembrator (Fisher Scientific, Pittsburgh, PA; medium intensity, 30 s). The homogenates were centrifuged for 10 min at 12,500g for 4°C. The supernatants were collected and protein concentrations were determined as described below. A nitrated protein solution was prepared for use as a standard by adding 8 μl (2 μl x 4) of chemically synthesized peroxynitrite anion (ONOO-) (concentration, 100–120 mM) to 3 ml of 0.04% (0.4 mg/ml) BSA in PBS. The amount of nitrotyrosine present in the peroxynitrite-treated BSA solution was measured at 430 nm (εmax = 4400 M−1 cm−1) using a SpectraMax-Plus microplate spectrophotometer (Molecular Devices) and expressed as nanograms per milligram of protein. This study was supported by grants from the National Institutes of Health.
nitrotyrosine concentration, 0.75–75 ng/ml). These standard samples, along with tissue samples from hearts (protein concentration, 4 mg/ml) were applied to disposable sterile enzyme-linked immunosorbent assay plates (Corning Glassworks, Corning, NY) and incubated overnight at 4°C. The plate was then washed with 200 µl of PBS/0.1% Tween buffer, followed by incubation with heat-inactivated 10% goat serum in PBS for 1 h at 37°C to block nonspecific binding. The primary antibody (rabbit polyclonal antibody against nitrotyrosine from Dr. J. Beckman, University of Alabama at Birmingham, Birmingham, AL: 1:2000 dilution, incubation for 2 h at 37°C) and secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:3000 dilution, incubation for 1 h at 37°C) were added, and the peroxidase reaction product was then generated using 2.2 mM 3,3′-diaminobenzidine tetrahydrochloride solution (Abbott Diagnostics, Abbott Park, IL). The plate was incubated for 20 min in the dark at room temperature and the reaction was stopped by addition of 100 µl of 2 M H2SO4 to each well. The optical density was measured at 430 nm with a SpectraMax-Plus microplate spectrophotometer (Molecular Devices). The amount of nitrotyrosine content in tissue samples was calculated using standard curves generated from nitrated BSA containing known amounts of nitrotyrosine, and expressed as picomoles per milligram of protein.

Myocardial Lipid Peroxidation. Lipid peroxidation was determined by the formation of thiobarbituric acid reactive substances (TBARs) (Kirshenbaum and Singal, 1993). Briefly, myocardial tissue was homogenized in 9 volumes of 1.15% cold KCl solution over ice. To a 50-µl sample homogenate, 1.95 ml of assay buffer (0.25 ml of 3% sodium dodecyl sulfate, 0.75 ml of 20% acetic acid, 0.75 ml of 0.8% aqueous thiobarbituric acid, and 0.2 ml of Milli-Q water) were added, and the mixture was incubated at 95°C for 75 min. After cooling to room temperature, 0.5 ml of water was added, and the sample was then extracted with isovolumic (2.5 ml) butanol/pyridine (15:1). After vortexing, the sample was centrifuged at 4000 g for 10 min. The optical density of the upper organic layer was read spectrophotometrically at 532 nm.

Measurement of Protein

Protein concentrations of the homogenates were determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL). The protocol was prestored in a SpectraMax-Plus microplate spectrophotometer by the manufacturer.

Materials

All chemicals were purchased from Sigma Chemical unless otherwise stated. Glutathione monoethyl ester and ascorbic acid were freshly prepared daily. Male Sprague-Dawley rats (220–260 g) fed on a standard laboratory diet were used in this study. The experiments were performed in adherence to National Institutes of Health Guidelines on the Care and Use of Laboratory Animals and were approved by the Thomas Jefferson University Committee on Animal Care.

Statistical Analysis

All data in the text, figures, and tables are presented as means ± S.E. of n independent experiments. Incidence of VF among the groups was compared by chi square analysis. Hemodynamic data were analyzed by two-way analysis of variance and all other data were analyzed with one-way analysis of variance followed by the Bonferroni correction for post hoc t tests (GraphPad Prism; GraphPad Software, San Diego, CA). Probabilities of 0.05 or less with Bonferroni correction were considered statistically significant.

Results

Effect of Ascorbic Acid and GSHme Treatment on Myocardial GSH Content. To determine whether 30 min of ischemia followed by 120 min of reperfusion in isolated perfused rat hearts may reduce myocardial GSH, and whether treatment with GSHme may restore myocardial GSH content, we measured myocardial GSH contents in all experimental groups. Myocardial ischemia and reperfusion resulted in a significant reduction in GSH content (9.8 ± 1.58 versus 16.1 ± 1.04 nmol/mg of protein in sham MI hearts, P < 0.01). Treatment with ascorbic acid had no effect on ischemia-reperfusion-induced GSH depletion, but administration of GSHme at the onset of reperfusion increased GSH content in a concentration-dependent manner (12.2 ± 0.99, 13.1 ± 0.87, and 14.2 ± 0.99 nmol/mg of protein with GSHme concentrations of 0.5, 1, and 2 mM, P < 0.01 versus vehicle). Combination of ascorbic acid and GSHme at 1 mM each also increased GSH content to a level that was comparable to that observed with 1 mM GSHme alone (Fig. 1). These results
demonstrated that administration of GSHme effectively restored intracellular GSH level, and coadministration of ascorbic acid had no effect on intracellular GSH restoration by GSHme.

**GSHme, but not Ascorbic Acid, Exerted Significant Cardioprotection in a Dose-Dependent Manner When Administered Alone.** In our first series of study, GSHme or ascorbic acid was administered alone at different concentrations to determine the optimal dose at which GSHme or ascorbic acid may exert a significant protection against reperfusion injury. As summarized in Fig. 2, administration of ascorbic acid alone at a concentration up to 2 mM exerted no significant protection either on cardiac function recovery or myocardial creatine kinase loss. In contrast, administration of GSHme alone improved cardiac function recovery and reduced myocardial creatine kinase loss in a dose-dependent manner. The protective effects of GSHme reached its plateau at 1 mM, and further increasing GSHme concentration to 2 mM only slightly increased its protective effects. Therefore, 1 mM GSHme was used in all subsequent experiments in determining the synergistic effects between GSHme and ascorbic acid.

**Effects of Different Treatments on Heart Rate Recovery, and Incidence of Ventricular Tachyarrhythmias and Fibrillation after Reperfusion.** In our isolated KH-perfused rat heart model, heart rates ranged from 253 to 309 bpm before ischemia. There was no significant difference among the groups studied. In hearts subjected to sham myocardial ischemia-reperfusion, heart rates were slightly decreased at the end of the 2-h perfusion period (271 ± 4.8–260 ± 4.9 bpm). All hearts lost their spontaneous beating within 5 min of ischemia and gradually recovered after reperfusion. At 60 min of reperfusion, heart rates recovered to 214 ± 6.3 bpm in the vehicle-treated group. Treatment with GSHme or ascorbic acid alone failed to improve heart rate recovery (221 ± 7.1 and 217 ± 6.9 bpm, respectively, \( P > 0.05 \)). However, when hearts were treated with a combination of GSHme and ascorbic acid, heart rates were recovered to a significantly higher level compared with vehicle (233 ± 4.4 bpm, \( P < 0.05 \) versus vehicle).

Global ischemia followed by reperfusion frequently resulted in the induction of episodes of ventricular tachyarrhythmias that occurred mostly in the first 5 min of reperfusion. The majority of hearts reverted to a stable sinus rhythm within 15 min of reperfusion. In 10 hearts receiving vehicle, four hearts developed VF that reverted to normal sinus rhythm within 1 min by a gentle tap with a small forceps handle. Although treatment with GSHme alone slightly reduced the incidence of VF, the only treatment that significantly reduced the incidence of VF was the combination of 1 mM GSHme and 1 mM ascorbic acid. No VF was observed during the entire reperfusion period in this group (Table 1).

**Effects of GSHme, Ascorbic Acid, or Their Combination on Cardiac Contractile Function Changes after Ischemia and Reperfusion.** At the end of the 15-min equilibration period (immediately before ischemia), LVDP and LVdP/dt\(_{\text{max}}\) ranged from 82 to 110 mm Hg and 2210 to 2515 mm Hg/s, respectively. There were no significant differences in these two functional parameters among the groups (Fig. 3). Thirty minutes of global ischemia followed by reperfusion caused a severe cardiac dysfunction manifested by elevated LVEDP, decreased LVSP and LVDP, and reduced LVdP/dt\(_{\text{max}}\) (Fig. 3). Administration of ascorbic acid alone failed to improve LVDP and LVdP/dt\(_{\text{max}}\) recovery. In contrast, administration of GSHme alone significantly increased LVDP at 30 and 60 min after reperfusion (Fig. 3). This protection, however, diminished to a level that was not statistically significant at 120 min after reperfusion. Most interestingly, although administration of 1 mM ascorbic acid alone had no protective effects against ischemia-reperfusion-induced cardiac dysfunction, coadministration of ascorbic acid with GSHme exerted more profound and sustained protection in improving cardiac function recovery compared with treatment with 1 mM GSHme alone (Fig. 3). Thus, at 120 min of reperfusion, LVDP and LVdP/dt\(_{\text{max}}\) were not only significantly higher than that in the vehicle group but also significantly higher than that observed in hearts treated with GSHme alone (Fig. 3). These results suggested that GSHme and ascorbic acid exerted significant cardioprotective effects in a synergistic manner.

**Effects of GSHme, Ascorbic Acid, or Their Combination on Cardiac Cellular Injury after Ischemia and Reperfusion.** To examine the effects of different antioxidant treatments on cellular injury, we measured myocardial creatine kinase isoenzyme loss after 30 min of ischemia and 120 min of reperfusion. As illustrated in Fig. 4, ischemia-reperfusion resulted in significant myocardial creatine kinase loss. The administration of 1 mM GSHme alone at the onset of reperfusion significantly attenuated myocardial creatine kinase loss (Fig. 4). In contrast, the administration of ascorbic acid alone exerted no significant protective effect. However, when 1 mM ascorbic acid (a nonprotective concentration when administered alone) was administrated together with 1 mM GSHme, a marked reduction of myocardial creatine kinase loss was observed (Fig. 4). The myocardial creatine kinase content in this group was significantly higher than in those hearts treated with 1 mM GSHme alone (\( P < 0.05 \)).

To determine whether the combination of ascorbic acid and GSHme is indeed required to exert the optimal cardioprotection, another two groups of animals were studied in which higher concentrations of ascorbic acid or GSHme (2 mM) were administered alone. The data showed that further increasing ascorbic acid to 2 mM failed to increase its protec-
tion (Fig. 2). Although increasing GSHme to 2 mM slightly enhanced its cardioprotection (Fig. 2), this treatment failed to restore myocardial creatine kinase content to a level that was comparable to that seen in the hearts treated with a combination of ascorbic acid and GSHme at a lower concentration (myocardial creatine kinase content: 6.1 ± 0.21 IU/mg of protein, P < 0.05 versus GSHme alone at 2 mM). These results further indicate that there was a synergistic effect of GSHme and ascorbic acid in protecting myocardial tissue from reperfusion injury.

Effects of GSHme, Ascorbic Acid, or Their Combination on Myocardial Lipid Peroxidation after Reperfusion. To determine whether antioxidant treatment may reduce myocardial lipid peroxidation, thus attenuating myocardial reperfusion injury and improving cardiac functional recovery, we performed additional experiments in which hearts were treated as described above and lipid peroxidation was assessed by measuring myocardial TBAR formation. In hearts subjected to ischemia and reperfusion and treated with vehicle, myocardial TBAR contents were markedly increased (5.69 ± 0.65 nmol/mg of protein versus 2.13 ± 0.11 nmol/mg of protein in sham MI hearts, P < 0.01). The administration of ascorbic acid or GSHme alone only slightly reduced myocardial TBAR contents compared with vehicle (P > 0.05) (Fig. 5). However, when ascorbic acid and GSHme were combined, myocardial TBAR content was reduced to a level that was significantly lower than that seen in hearts treated with vehicle. These results demonstrated that administration of a nonprotective concentration of ascorbic acid with GSHme enhanced the protective effect of GSHme in reducing ischemia-reperfusion-induced lipid peroxidation.

Effects of GSHme, Ascorbic Acid, or Their Combination on Myocardial Nitrotyrosine Contents after Reperfusion. Recent experimental evidence suggests that ONOO\(^-\), the reaction product of nitric oxide and superoxide anion, can result in significant lipid peroxidation and cellular injury (Beckman and Koppenol, 1996). To determine whether treatment with GSHme, ascorbic acid, or their combination reduced ONOO\(^-\) formation, thus attenuating myocardial lipid peroxidation and myocardial injury, we measured myocardial nitrotyrosine content, the footprint of ONOO\(^-\) formation. Our data demonstrated that ischemia-reperfusion resulted in a 2.6-fold increase in nitrotyrosine concentration (2.69 ± 0.18 versus 1.01 ± 0.21 pmol/mg of protein), indicating that there was significant peroxynitrite formation. Treatment with GSHme moderately reduced myocardial nitrotyrosine levels (P < 0.05). Most interestingly, although administration of ascorbic acid alone had no effect on myocardial nitrotyrosine content, coadministration of 1 mM ascorbic acid with 1 mM GSHme reduced myocardial nitrotyrosine content to a level that was significantly lower than that observed in the GSHme-treated group (Fig. 6).

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham + Vehicle</th>
<th>MI + Vehicle</th>
<th>MI + GSH1</th>
<th>MI + GSH2</th>
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<td>1/10</td>
<td>4/10</td>
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<td>0/10*</td>
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AA, ascorbic acid.

*P < 0.05 versus vehicle.

**Fig. 3.** Effects of ascorbic acid, GSHme, or their combination on cardiac function (LVDP and LVDp/dt\(_{max}\)) and coronary flow rate (CFR) recovery after 30 min of global ischemia and 120 min of reperfusion. *, P < 0.05; ***, P < 0.01 versus vehicle; #, P < 0.05; ##, P < 0.01 versus GSHme alone.
Using an isolated perfused heart model, we have demonstrated that 1) pharmacological concentrations of GSHme, but not ascorbic acid, exerted significant cardiac protective effects against ischemia-reperfusion injury when administered at the onset of reperfusion; and 2) although the administration of ascorbic acid alone failed to exert protective effects on any observed parameters, coadministration of ascorbic acid with GSHme markedly enhanced protection exerted by GSHme. To our knowledge, these results demonstrated for the first time that GSHme and ascorbic acid exert cardioprotective effects against myocardial reperfusion injury in a synergistic manner and that the superior protection exerted by GSHme and GSHme plus ascorbic acid is due, at least in part, to their effect in reducing peroxynitrite formation in postischemic myocardial tissue.

Substantial evidence indicates that the balance between oxidants and antioxidants is severely disturbed in ischemic reperfused myocardial tissues. Generation of reactive oxygen species, including superoxide (\( \cdot \)O\(_2\)) hydrogen peroxide (H\(_2\)O\(_2\)), and hydroxyl radical (\( [\text{rchem}]\)OH), is markedly increased after reperfusion (Zweier et al., 1987; Bolli, 1991). Production of reactive nitrogen species, including ONOO\(^-\), nitroxyl anion (NO\(^-\)), and NO\(^*\) free radical, has also been reported to be increased in ischemic reperfused tissues (Zweier et al., 1995; Wang and Zweier, 1996; Ma et al., 1999). On the other hand, endogenous antioxidants, including enzymatic free radical scavengers and nonenzymatic antioxidants, are significantly reduced after ischemia and reperfusion (Dhalla et al., 2000). In a recent study, Haramaki et al. (1998) further demonstrated that cardiac antioxidants were changed in a systematically organized relationship under ischemia and reperfusion. Myocardial hydrophilic antioxidants such as ascorbic acid and GSH were oxidized first in ischemic reperfused myocardial tissue, but antioxidant enzymes and lipophilic antioxidants remained unchanged unless excess H\(_2\)O\(_2\) was added. Based on these findings, it was proposed that a strategy to maintain myocardial hydrophilic...
Antioxidants may lead to clinically relevant antioxidant treatment in the future. In this connection, a previous study by Ferrari et al. (1991) demonstrated that administration of N-acetylcysteine before myocardial ischemia (but not at the onset of reperfusion) effectively increased intracellular GSH concentration and reduced postischemic myocardial injury. In contrast, administration of GSH had no effect on intracellular GSH concentrations, and failed to exert significant protection against myocardial reperfusion injury (Ferrari et al., 1991). Because N-acetylcysteine had to be administered before the onset of ischemia to exert its cardioprotective effects, this treatment has limited clinical application (Ferrari et al., 1991).

In the present study, we have demonstrated that administration of glutathione monoethyl ester, a cell membrane-permeable form of GSH (Anderson et al., 1985), at the onset of reperfusion reduced myocardial reperfusion injury in a concentration-dependent manner. To our surprise, the administration of ascorbic acid, another hydrophilic antioxidant, by an identical regimen failed to exert significant protection in this ischemic reperfusion model. Previous studies have demonstrated that GSH and ascorbic acid have many actions in common and that they can compensate for each other under conditions where one of them is systemically depleted (Martenson et al., 1991, 1993; Meister, 1994). However, our present data argue against GSH and ascorbic acid playing equally important roles in myocardial ischemia and reperfusion, a pathological condition where both GSH and ascorbic acid have been reduced locally in ischemic reperfused tissue.

The superior protective effects of GSHme are likely related to its unique antioxidant properties that are not shared by ascorbic acid. First, functioning as a substrate of glutathione peroxidase, GSH (but not ascorbic acid) plays a pivotal role in removing H₂O₂ and preventing the generation of highly toxic *OH, thus reducing ROS-induced myocardial injury. Second, recent studies have demonstrated that GSH acts as an ONOO⁻ scavenger, and that the reaction of GSH with ONOO⁻ forms an S-nitrosothiol compound that regenerates *NO over a prolonged period (Wu et al., 1994; Moro et al., 1995; Nakamura et al., 2000). Therefore, the administration of GSHme, but not ascorbic acid, may convert a cytotoxic agent, ONOO⁻, to a cytoprotective agent, *NO, thus reducing reactive nitrogen species-caused myocardial injury after ischemia and reperfusion. In this connection, the present study provides direct evidence that treatment with GSHme significantly increased myocardial GSH levels and reduced myocardial nitrotyrosine concentration, indicating that this treatment indeed reduced the ONOO⁻ concentration in postischemic myocardial tissue. Finally, in the present experiment, we have administered GSHme, a form that is easily transported into the cells and that increases intracellular reduced GSH (Meister, 1994). In contrast, although reduced ascorbic acid was added to the perfusion solution, intracellular accumulation of ascorbic acid may not have been increased by this treatment. Considerable evidence indicates that dehydroascorbate is a major transport form of ascorbic acid across cell membranes (Vera et al., 1993; Washko et al., 1993; Meister, 1994; Van Duijn et al., 1998). In many cells, accumulation of ascorbic acid is achieved through a facilitative glucose transporter, GLUT-1 (Vera et al., 1994). This transporter efficiently transports dehydroascorbate into the cell, but not ascorbic acid itself. Inside the cell, dehydroascorbate is reduced to ascorbic acid by other reducing agents, such as GSH. Under pathological conditions where total reducing capacity in the cells is markedly reduced, as in myocardial ischemia and reperfusion, dehydroascorbate might not be able to be reduced to ascorbic acid, thus limiting its cytoprotective effects against reperfusion injury.

The most interesting finding of the present study is that although administration of 1 mM ascorbic acid alone exerted no protection against reperfusion injury, coadministration of ascorbic acid with GSHme exerted significantly greater protection than GSHme alone. This synergistic effect between these two functionally similar antioxidants provides further supporting evidence that the intracellular antioxidant defense machinery is organized as a network and functions as an integrated system. It is generally accepted that GSH, ascorbic acid, and α-tocopherol operate as a reaction chain to ensure maximum membrane protection against oxidative damage (Chan et al., 1991). GSH, either exogenously supplied or endogenously regenerated from oxidized glutathione by glutathione reductase,
is one of the most important reducing agents in the regeneration of ascorbic acid from its oxidized form, dehydroascorbate. Ascorbic acid, in turn, regenerates α-tocopherol from the tocopheroyl radical and thus assists in recycling α-tocopherol (Maxwell and Lip, 1997). α-Tocopherol is the major lipophilic antioxidant present in the cell membrane, where it protects vulnerable membrane polyunsaturated fatty acids from the free radical-initiated chain reactions that ultimately lead to lipid peroxidation and membrane disruption. Therefore, the administration of ascorbic acid alone may not increase intracellular ascorbic acid when intracellular GSH is significantly decreased, because dehydroascorbate, rather than ascorbic acid itself, is selectively transported into the cell. However, when ascorbic acid is administered together with GSHme, increased intracellular dehydroascorbate can be reduced to ascorbic acid, thereby reestablishing a complete antioxidant chain and achieving best protection. In support of this conclusion, we have directly demonstrated in the present study that the administration of either GSHme or ascorbic acid alone at 1 mM had no significant effect on myocardial TBAR content, a well accepted index for lipid peroxidation. When both ascorbic acid and GSHme were administered in combination, myocardial TBAR content was decreased, myocardial membrane integrity was maintained, myocardial infarct size was reduced, and cardiac function was improved. These results may form a foundation in the search for the optimal therapeutic regimens, including cocktail treatments, against myocardial ischemia-reperfusion injury.

Limitations. It has been recently reported that, under physiological conditions, dehydroascorbate uptake via facilitated-diffusion glucose transporters is unlikely to play a major role in the uptake of vitamin C due to the high concentrations of glucose that will effectively block influx (Li et al., 2001). In addition, ascorbic acid has been reported to enter cells via Na+-dependent systems, and two isoforms of these transporters (SVCT1 and SVCT2) have recently been cloned from humans and rats (Liang et al., 2001). In the present study, intracellular dehydroascorbate and ascorbic acid were not directly measured due to our methodological limitation. Therefore, the proposed mechanisms by which GSH and ascorbic acid exert their synergistic effects are largely speculative, although they are likely to exist based on results from other studies. Moreover, both ascorbic acid and GSH have been reported to function as chelator of copper, although the role of copper in reperfusion injury is not clear and the effects of copper chelators on reperfusion injury has never been previously studied. Finally, using a similar experimental procedure commonly used by many other investigators, we immersed hearts in a 50-ml chamber filled with KH buffer at 37°C. Therefore, a diffusion limited supply of glucose and oxygen to the hearts may have occurred during global ischemia. However, this limitation should not alter our conclusion because this phenomenon should occur with all hearts studied. Nevertheless, our present study provides direct evidence that the protective effects of GSH cannot be replaced by ascorbic acid under the condition of myocardial ischemia-reperfusion. This is different from a condition in which GSH is systemically depleted, and the antioxidant function of GSH is largely replaced by exogenous administration of ascorbic acid.

References


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