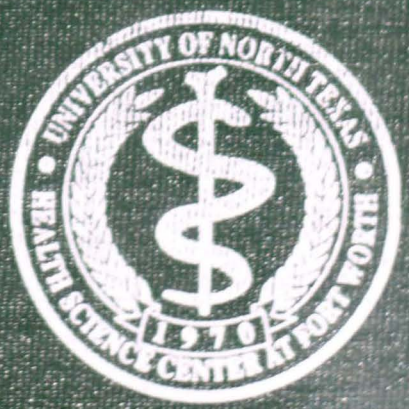
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Squires, Jeffrey E., *Acetoacetate: A Cardioprotective Antioxidant.* Master of Science, June, 2002, 100 pp., 1 table, 18 illustrations, bibliography, 70 titles.

The purpose of this study was to test the effectiveness of acetoacetate and β–hydroxybutyrate as myocardial protectants following peroxide injury and to determine acetoacetate's ability to potentiate β–adrenergic responsiveness following ischemia-reperfusion injury. This study utilized antegradely perfused isolated working hearts excised from male guinea pigs and sustained with glucose-fortified Krebs-Henseleit. Hearts were challenged by either 10 min perfusion with 100 μM H_2O_2 or 45 min of low flow ischemia exacerbated by *L*-norepinephrine infusion. H_2O_2-challenged hearts were treated with 5 mM acetoacetate or β–hydroxybutyrate, whereas hearts injured by ischemia/reperfusion were treated with 5 mM acetoacetate. In the case of the ischemically injured hearts, acetoacetate treatment was combined with 2 nM isoproterenol to delineate acetoacetate's ability to enhance β–adrenergic responsiveness to submaximal inotropic stimulation. Data were compared to non-injured time control hearts and injured untreated hearts to determine the impact of ketone body treatment. Acetoacetate increased citrate and glucose 6-phosphate content, nearly restored power, and increased the glutathione antioxidant redox potential (GSH/GSSG) by 140% in H_2O_2-injured myocardium. Although β–hydroxybutyrate increased citrate, an activator of NADPH-generating pathways, and glucose 6-phosphate, the substrate for the hexose monophosphate shunt to the same extent as acetoacetate, β–hydroxybutyrate raised GSH/GSSG by only 60% and did not enhance cardiac power. Therefore, acetoacetate enhances contractile function by augmenting the glutathione redox potential, and does so

by additional mechanisms independent of the citrate and hexose monophosphate pathway. In hearts stunned by ischemia/reperfusion, acetoacetate and isoproterenol each increased power and glutathione redox potential three-to-fourfold, but phosphocreatine potential was 70% higher in acetoacetate hearts. Combined, acetoacetate + isoproterenol synergistically increased power and GSH/GSSG 16- and 17- fold respectively, doubled {$NADPH/NADP^+$}, and increased cyclic AMP content 30%. These finding support the conclusion that acetoacetate enhances myocardial sensitivity to β–adrenergic stimulation possibly by enhancing GSH/GSSG.

ACETOACETATE: A CARDIOPROTECTIVE ANTIOXIDANT

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ACETOACETATE: A CARDIOPROTECTIVE ANTIOXIDANT

THESIS

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CHAPTER I

INTRODUCTION

Cardiac stunning and myocardial prooxidant injury. Myocardial stunning develops upon coronary reperfusion following a brief episode of ischemia and results in reversible cardiac injury characterized by modest structural damage [8] and functional depression [6]. Reactive oxygen species, generated during ischemia and in a massive burst upon reperfusion, have been implicated in the pathogenesis of cardiac stunning. Hydrogen peroxide (H_2O_2), superoxide anions ($\cdot O_2$), hydroxyl radicals ($\cdot OH$), and peroxynitrite ($ONOO^-$) [2, 18] react with cellular components causing enzyme deactivation, protein denaturation, and lipid peroxidation [7]. The structural modifications inflicted by oxyradicals are a cause of cardiomyocyte dysfunction during cardiac stunning [6].

Inotropic responses to β–adrenergic stimulation are compromised in stunned myocardium [24, 4, 66]. β–adrenergic impairment is likely due to oxidative damage to protein components of the β–adrenergic signaling cascade, which dampens myocardial responses [55] to low, submaximal concentrations of the β–adrenergic agonist isoproterenol [65]. Ischemia/reperfusion blunts cardiac inotropic responses by reducing the binding affinity and density of β_1–adrenergic receptors, by inactivating adenylate cyclase [55] through the oxidation of sulfhydryl groups within the enzyme [23], by inactivating sarcoplasmic

reticular Ca^{2+} ATPase [13], and by decreasing the cAMP binding affinity to protein kinase A [42].

Perfusing the heart with relatively low doses of hydrogen peroxide (~100μM H_2O_2) simulates the oxidative stress associated with cardiac stunning [29] by causing cytosolic calcium overload [40], sarcolemmal disruption [38], depletion of endogenous antioxidant defenses [41] and impairment of glycolytic [29, 14, 37] and oxidative metabolism [29, 64]. Although H_2O_2 itself is relatively harmless at low concentrations *in vivo* [33], the highly cytotoxic hydroxyl radical can be formed from H_2O_2 and superoxide ($\cdot O_2^-$) by the Fenton [25] and Haber-Weiss [32] reactions. $\cdot O_2^-$ is generated by single-electron reduction of O_2 by semiubiquinone in the mitochondrial respiratory chain, xanthine oxidase, arachidonic acid metabolism, and from H_2O_2 by reversal of the superoxide dismutase reaction [49]. $\cdot O_2^-$ spontaneously combines with nitric oxide (NO·) to form peroxynitrite ($ONOO^-$), a cytotoxic reactive intermediate [59] (Figure 1).

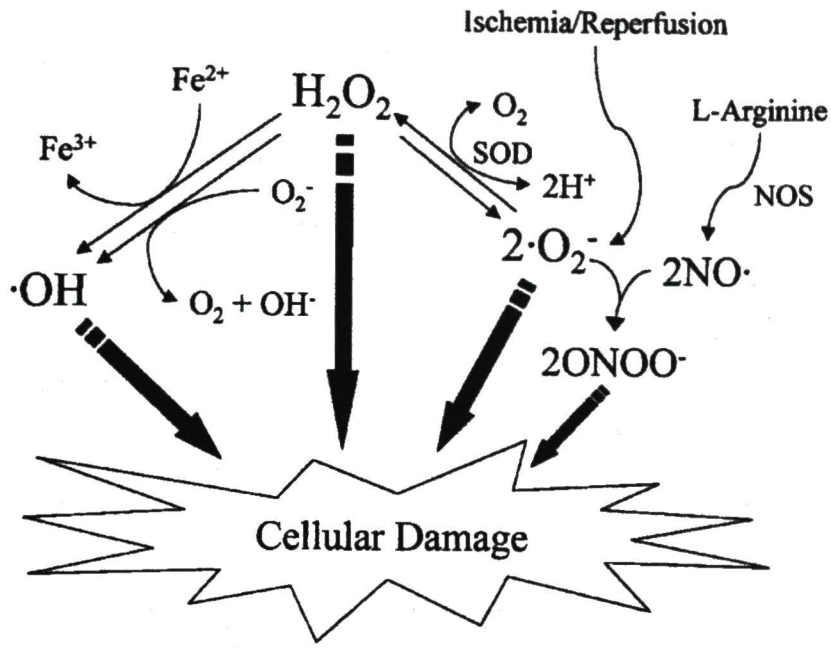

Figure 1. *Reactive oxygen species formation*

Perfusion of isolated rat hearts with low doses of H_2O_2 (50-500μM) impairs carbohydrate metabolism [37]. H_2O_2 inactivates glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [14], blunts glycolytic flux, and causes accumulation of fructose 1,6-bisphosphate and triose phosphate intermediates proximal to GAPDH in the glycolytic sequence [11]. Glycolytic sugar phosphates accumulate at the expense of ATP and activate the hexose monophosphate shunt at the onset of H_2O_2 induced stress in cultured neonatal rat cardiomyocytes [37]. Peroxide inhibits pyruvate dehydrogenase, but to a lesser extent than GAPDH, so the entry of acetyl CoA derived from carbohydrate fuels into the TCA cycle is less restricted than the production of ATP through glycolysis [37]. H_2O_2 also inactivates the mitochondrial enzymes ATP synthase, adenine nucleotide translocase, and NADH-CoQ reductase, thereby compromising the major energy-generating metabolic pathways in the myocardium [64].

Endogenous antioxidant defenses: the glutathione system. Glutathione (GSH), a tripeptide synthesized from glutamate, glycine, and cysteine in the γ–glutamyl cycle [51], acts as the cardiomyocyte's central defense against oxidative stress [41, 22]. The glutathione redox potential is in chemical equilibrium with the other cellular redox systems including the nicotinamide adenine dinucleotide phosphate ($NADPH/NADP^+$), thioredoxin, and α-tocopherol systems [61]. Accordingly, the ratio between GSH and its oxidized derivative glutathione disulfide (GSSG) serves as an accurate index of oxidative damage [26] and an integrated measure of the cellular redox state [61]. GSH directly neutralizes H_2O_2 and phospholipid peroxides in the glutathione peroxidase reaction yielding GSSG and H_2O [62]. GSSG is recycled to GSH by glutathione reductase utilizing reducing equivalents provided by NADPH produced by the hexose monophosphate shunt and isocitrate dehydrogenase.

Glutathione exists in dynamic equilibrium with sulfhydryl groups in cellular proteins [26]. By supplying reducing equivalents for thiol-disulphide exchange reactions catalyzed by thioltransferases [47], GSH maintains these sulfhydryls in their reduced state and, thus, preserves protein activity. Elevated intracellular GSSG also modifies proteins via the thioltransferase reaction forming mixed disulfides [48]. Intracellular GSSG concentration is regulated. GSSG translocases export accumulated GSSG from cells [62] as a means of maintaining intracellular GSH redox potential during oxidative stress.

Antioxidant actions of pyruvate. Pyruvate, an α-keto acid and natural cardiac fuel, acts as an antioxidant in peroxide-injured [46] or postischemic stunned [65] isolated perfused guinea-pig hearts by several mechanisms. As an α-keto acid, pyruvate scavenges and neutralizes H_2O_2 and lipid peroxides in a direct, nonenzymatic chemical reaction [21]. Pyruvate also could bolster endogenous antioxidants by increasing myocardial citrate content. Anaplerotic pyruvate carboxylation generates 4-carbon TCA cycle intermediates leading to citrate accumulation which increases flux through two NADPH-generating metabolic pathways. Citrate inhibits phosphofructokinase [28] and diverts glucose 6-phosphate into the hexose monophosphate shunt which yields two NADPH per glucose 6-phosphate consumed. By elevating the $NADPH/NADP^+$ ratio, the hexose monophosphate shunt drives the glutathione reductase reaction towards GSSG reduction and thereby enhances the cellular antioxidant potential. After its conversion to isocitrate, citrate may also activate NADPH production by $NADP^+$ dependent isocitrate dehydrogenase.

Pyruvate potentiates β–adrenergic inotropism in stunned myocardium submaximally stimulated with 2 nM isoproterenol [66]. Pyruvate likely augments the myocardium's β–adrenergic response by enhancing the glutathione redox potential and possibly by increasing the cellular phosphocreatine phosphorylation potential [66], a measure of the cytosolic ATP phosphorylation potential and Gibbs free energy of ATP hydrolysis [68]. By elevating myocardial GSH/GSSG, pyruvate may support the restoration of cellular protein

components damaged by stunning and thereby restore the myocardium's responsiveness to isoproterenol [65].

Acetoacetate and β–hydroxybutyrate: two hypothetical antioxidants. Ketone bodies such as acetoacetate, a β-keto acid, and its reduced congener β–hydroxybutyrate, possess chemical structures resembling pyruvate and may act as antioxidants in the heart by similar mechanisms. Within the cell, β–hydroxybutyrate is oxidized to form acetoacetate by β–hydroxybutyrate dehydrogenase. Acetoacetate is then converted to acetoacetyl-CoA by 3-ketoacyl-CoA transferase which transfers a coenzyme-A unit from succinyl-CoA to the keto-acid thereby forming a thioester. Acetoacetyl-CoA is then cleaved by thiolase yielding two acetyl-CoAs (Figure 2). The conversion of acetoacetate and β–hydroxybutyrate to acetyl-CoA would inhibit pyruvate dehydrogenase [56], thereby diverting pyruvate to carboxylation pathways catalyzed by pyruvate carboxylase and malic enzyme. These mechanisms would anaplerotically generate citrate and thereby enhance the glutathione antioxidant potential by generating NADPH.

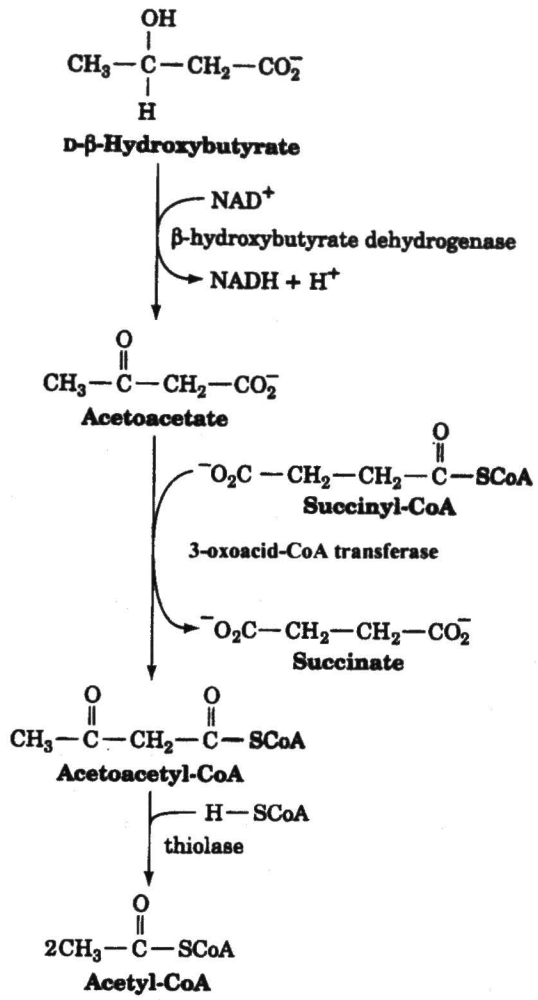

Figure 2. *The metabolic conversion of acetoacetate and β-hydroxybutyrate to acetyl-CoA*

In addition to the metabolic mechanisms described above, acetoacetate and β–hydroxybutyrate may support more direct antioxidant mechanisms. Acetoacetate absorbs peroxynitrite in an aliphatic nitration reaction yielding the nonreactive derivative 2-nitroacetoacetate [67]. Acetoacetate may also promote antioxidation through its conversion to acetoacetyl-CoA by 3-ketoacyl-CoA transferase. Indeed, isolated rat hearts perfused with 10 mM acetoacetate generated a sevenfold increase in acetoacetyl-CoA

content [52]. Within the heart, acetoacetyl-CoA directly neutralizes H_2O_2, generating an unknown reactive oxygen species which is subsequently neutralized by electrons donated by NADH [34].

Acetoacetate's putative antioxidant actions may mediate potentiation of β–adrenergic inotropism in stunned heart, similar to the recently reported effect of pyruvate [65, 66]. The elevation of reduced glutathione by acetoacetate may maintain or restore protein sulfhydryls and rectify structural modifications of key proteins associated with the β–adrenergic signaling cascade. Indeed, enhancement of the glutathione antioxidant redox state maintains the activity of adenylate cyclase [54] and may modulate the activity of other components associated with adrenergic stimulation that are known to be inactivated by stunning. Acetoacetate's conversion to the free radical scavenger acetoacetyl-CoA and its ability to directly neutralize peroxynitrite may directly alleviate oxidative stress imposed on the heart and thereby unburden the myocardium's endogenous glutathione defenses enabling further rectification of damaged cell structures. The hypothetical mechanism of acetoacetate's antioxidant action is outlined below (Figure 3).

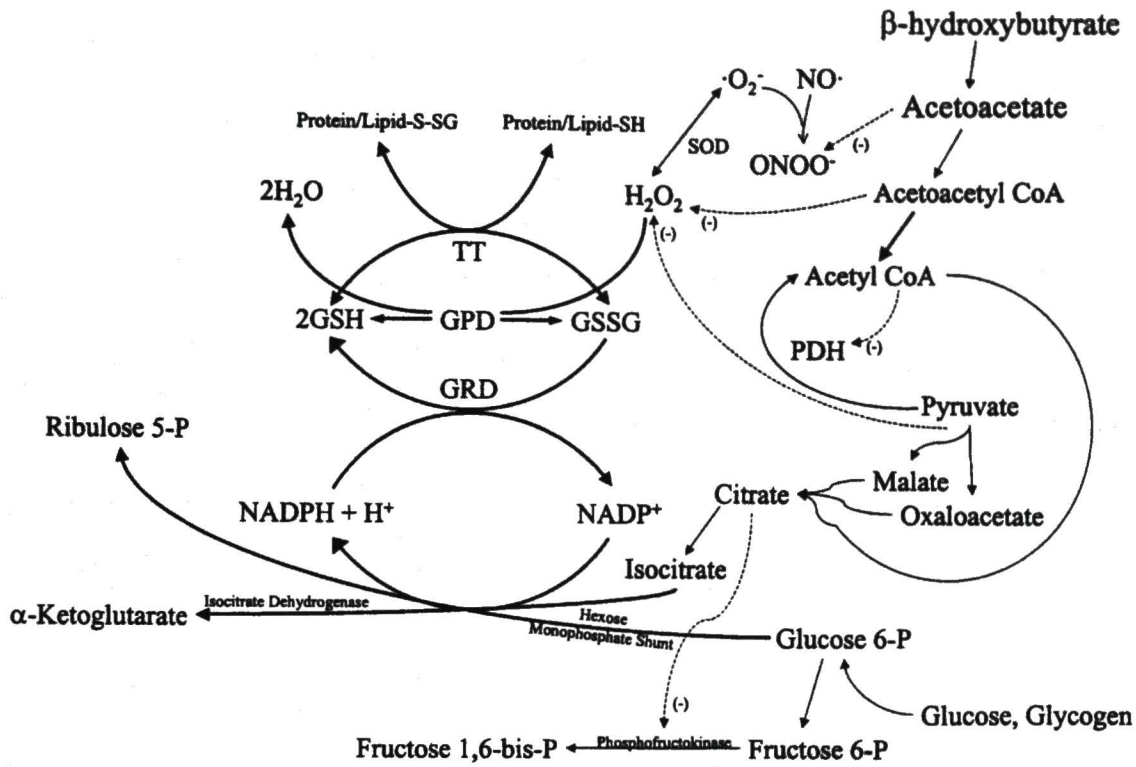

Figure 3. *Hypothetical mechanism of acetoacetate's antioxidative action*

Hypotheses. Two hypotheses were tested in this investigation: 1. Acetoacetate and β–hydroxybutyrate restore antioxidant redox state and contractile performance of H_2O_2-injured myocardium; 2. Acetoacetate restores contractile performance and β–adrenergic inotropism in stunned myocardium.

Methods

Isolated Working Heart Model. Hearts (n=66) were excised from male albino Hartley guinea pigs (400-600g) and antegradely perfused as working organs with 10 mM glucose

fortified Krebs-Henseleit bicarbonate buffer maintained at 38°C (pH 7.4, 295 mOsm) and equilibrated with 95% O_2 : 5% CO_2 gas mixture. The media contained concentrations of the following solutes (expressed in mM): NaCl 116, $NaHCO_3$ 26, KCl 3.5, KH_2PO_4 1.2, $CaCl_2$ 1.0, and $MgSO_4$ 0.6. Media also contained 5 U/l bovine insulin. Heart rate (HR), aortic pressure (Pa), left atrial filling pressure (Pv), and cardiac output (the sum of aortic and coronary flows) were measured to determine cardiac function. Stroke work was determined from stroke volume and left ventricular developed pressure, i.e. Pa-Pv. Cardiac power was calculated as the product of stroke work and HR. A schematic of the isolated working heart setup is diagramed below (Figure 4).

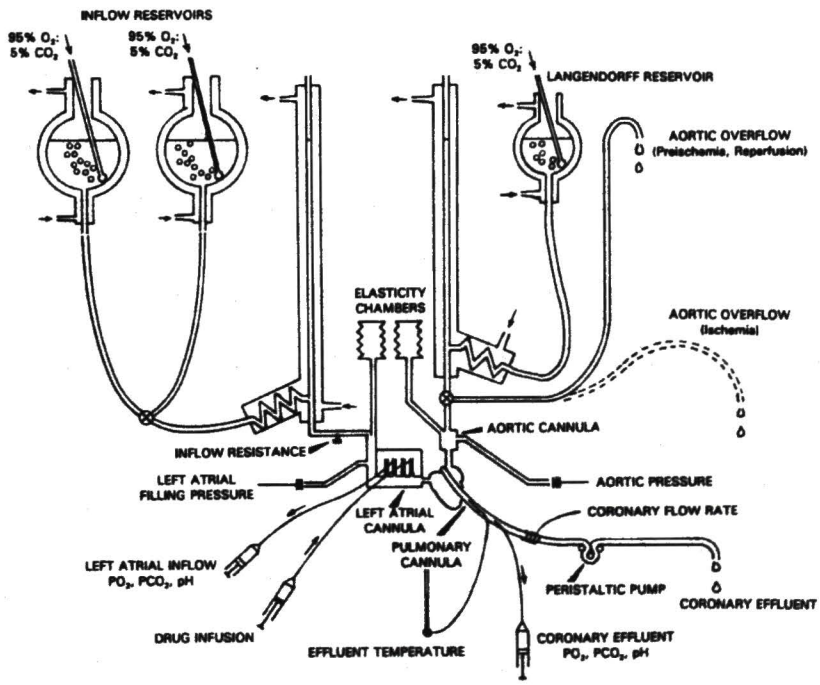

Figure 4. *Isolated heart perfusion system*

Hydrogen peroxide injury protocol. Following 15 min of normoxic perfusion with standard Krebs-Henseleit media hearts were challenged by 10 min perfusion with Krebs-Henseleit media containing 100 μM H_2O_2. Following peroxide injury, hearts were perfused for 30 min with H_2O_2-free Krebs-Henseleit media prior to initiation of 5 mM ketone body treatment with either acetoacetate or β-hydroxybutyrate. All perfusion media contained 10 mM glucose. Hearts were freeze-clamped at 60 min of treatment with nitrogen cooled Wollenberger tongs and stored at –90°C. Cardiac extracellular space was determined by continuous infusion of 300 mM sucrose as previously described (Itoya et al., 1996) for 5 min prior to freeze-clamping. The extracellular space was required to establish the intracellular inorganic phosphate concentration within the cardiomyocyte for calculating phosphorylation potentials.

Ischemia-reperfusion protocol to produce cardiac stunning. Following 15 min of preischemic baseline perfusion with standard Krebs-Henseleit media, hearts were hypoperfused by lowering the driving pressure for coronary perfusion. This was achieved by quickly lowering the aortic overflow column to drop the Pa from physiological pressure (90 cm H_2O) to 45 cm H_2O, then gradually lowering the column further to continuously lower Pa from 45 cm H_2O at a rate of 1 cm H_2O/min until a Pa of 12 cm H_2O was reached. To intensify ischemia, L-norepinephrine (~0.4 μM) was continuously infused throughout the 45 min hypoperfusion period. Left atrial filling pressure was maintained at 12 cm H_2O to prevent Frank-Starling mechanism dependent changes in cardiac work. After 45 min of ischemia, L-norepinephrine infusion was

discontinued and Pa restored to 90 cm H_2O by returning the aortic overflow column to its original height. The 15 min reperfusion period following ischemia preceded one of the two experimental protocols.

Metabolic and β–adrenergic treatment of stunned hearts. Four groups of stunned hearts were examined in the study: no treatment, 5 mM acetoacetate at 15-45 min reperfusion, 2 nM isoproterenol at 30-45 min reperfusion, and combined treatment with 5 mM acetoacetate at 15-45 min and 2 nM isoproterenol at 30-45 min reperfusion. Contractile performance and metabolic state of these hearts were compared with an untreated time control group perfused for 90 min but not subjected to ischemia and reperfusion. All hearts were freeze-clamped with liquid nitrogen cooled Wollenberger tongs at 90 min and stored in a –90°C freezer until metabolite extractions.

Myocardial Metabolites. The hearts were pulverized under liquid nitrogen. The powdered tissue was extracted for measurement of ATP, phosphocreatine (PCr), creatine (Cr), inorganic phosphate (Pi), $NADP^+$, NADPH, GSH, GSSG, citrate, glucose 6-phosphate and sucrose. Frozen tissue was placed in 4 vol. ice-cold perchloric acid (0.3 M) and homogenized with a precooled teflon piston for 1 min. The homogenate was then centrifuged (12000 × g) for ten minutes. The resulting supernatant was neutralized (pH 5.5-6.5) carefully using refrigerated KOH (1 M, 0.1 M) and kept at 0-4°C for 30 min, then centrifuged again. Metabolite concentrations in the extract supernatant were assayed in a Shimadzu model UV-1601PC dual wavelength uv/vis spectrophotometer (337nm

measuring wavelength, 417nm reference wavelength, ε=5.65 $M^{-1} \cdot cm^{-1}$). Myocardial cyclic AMP content was determined by competition-binding radioimmunoassay [39] (Appendix). Cyclic AMP content in myocardial extract was determined by comparison with a standard curve using Graph Pad Prism™ 2.0 software.

Statistical Analysis. Data are expressed as means ±SEM. Cardiac performance was analyzed using two-way ANOVA. Within-group treatment effects on cardiac performance were analyzed by comparing pre-treatment values measured at 28-30 min post H_2O_2 *vs.* values recorded at 60 min of treatment using paired Student's t-test. Coronary flows and resistances measured at 10 min H_2O_2 *vs.* 90 min post H_2O_2 in untreated hearts were compared by unpaired Student's t-test. Metabolites in the different groups were compared with one-way ANOVA. Within-group comparisons of data at different times in the experiment were accomplished with one-way ANOVA for repeated measures. Student-Newman-Keul's multiple comparison test was used *post hoc*. Statistical significance was assumed at $P < 0.05$.

CHAPTER II

Acetoacetate Augments Glutathione Redox State and Contractile Performance of Hydrogen Peroxide–Challenged Myocardium

Jeffrey E. Squires, Jie Sun, and Robert T. Mallet

ABSTRACT

Objective: Pyruvate, an α-keto carboxylate, mitigates hydrogen peroxide induced myocardial injury by directly neutralizing peroxides and by increasing metabolic flux through NADPH generating pathways to restore glutathione redox potential. Whether the natural β-keto carboxylate acetoacetate or its reduced congener β-hydroxybutyrate have similar antioxidant properties is unknown. This study tested the hypothesis that these ketone bodies restore antioxidant redox state and contractile performance of H_2O_2-injured myocardium. **Methods:** Krebs-Henseleit perfused working guinea-pig hearts, challenged by 10 min perfusion with 100 μM H_2O_2, were treated with 5 mM acetoacetate or β-hydroxybutyrate between 30 and 90 min post-H_2O_2. Hearts were subsequently snap-frozen for metabolite measurements. Phosphocreatine (PCr) phosphorylation potential and glutathione/glutathione disulfide ratio (GSH/GSSG) indexed myocardial energy state and antioxidant redox potential, respectively. **Results:** Cardiac power fell during and following H_2O_2 exposure before stabilizing at 30% of baseline. Acetoacetate nearly restored power, but β-hydroxybutyrate failed to improve cardiac performance. Neither compound significantly increased PCr potential. Acetoacetate bolstered GSH/GSSG by 140%, but β-hydroxybutyrate raised GSH/GSSG by a more modest 60%. Acetoacetate and β-hydroxybutyrate similarly increased citrate, an activator of NADPH-generating metabolism, and glucose 6-phosphate, the substrate for the hexose monophosphate shunt. **Conclusions:** Acetoacetate enhanced GSH redox potential and, thus, contractile performance of H_2O_2–injured myocardium, but citrate accumulation was not the lone mechanism for acetoacetate's antioxidant and cardiotonic actions.

Key words: contractile function, energy metabolism, free radicals, glycolysis, mitochondria

Abbreviations: AcAc: acetoacetate; β-OH-B: β-hydroxybutyrate; Cr: creatine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GSH: glutathione; GSSG: glutathione disulfide; NoTx: no treatment; P_a: aortic pressure; PCr: phosphocreatine; P_i: inorganic phosphate; P_v: left atrial filling pressure; TC: time control

INTRODUCTION

Reactive oxygen and nitrogen intermediates generated upon reperfusion of ischemic myocardium are a major cause of post-ischemic cardiomyocyte injury and have been implicated in the pathogenesis of cardiac stunning [1]. By modifying proteins and phospholipids, hydrogen peroxide (H_2O_2), superoxide anions ($\cdot O_2^-$), hydroxyl radicals ($\cdot OH$) and peroxynitrite ($ONOO^-$), impair metabolism and contractile performance of cardiac muscle [2, 3]. Indeed, exposure of myocardium to H_2O_2 recapitulates the stunning phenotype [4]. Moreover, H_2O_2 is a precursor of the highly cytotoxic $\cdot OH$ via the Fenton [5] and Haber-Weiss [6] reactions.

Hydrogen peroxide compromises cardiac performance, in part, by disrupting glycolytic and oxidative energy production. By inactivating glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [7-9], H_2O_2 blunts glycolytic flux, causing glycolytic intermediates proximal to GAPDH to accumulate at the expense of ATP [10]. H_2O_2 also inactivates pyruvate dehydrogenase [8, 11], TCA cycle enzymes [12], NADH-CoQ reductase, ATP synthase, and adenine nucleotide translocase [13]. These enzyme impairments compromise the major energy-generating metabolic pathways in the myocardium.

In myocardium, the principal H_2O_2 detoxifying mechanism employs glutathione peroxidase, which converts H_2O_2 to water through oxidation of glutathione (GSH) to glutathione disulfide (GSSG) [14, 15]. GSSG is recycled to GSH by glutathione

reductase, using reducing equivalents provided by NADPH. As the major intracellular redox buffer [16], GSH also powers thiol transferases to convert oxidized protein mixed disulfides to reduced sulfhydryls [14].

Pyruvate, an α-keto carboxylic acid and natural cardiac fuel, functions as an antioxidant in peroxide-injured [17] or postischemic stunned heart [18] by at least two mechanisms. Pyruvate reduces H_2O_2 to H_2O in a direct, non-enzymatic decarboxylation reaction [19]. Pyruvate is also carboxylated to form the TCA cycle intermediates malate and oxaloacetate, leading to steady-state increases in citrate content [20, 21]. By inhibiting phosphofructokinase [22], citrate diverts glycolytic flux into the NADPH-generating hexose monophosphate shunt. Also, the conversion of citrate to isocitrate supplies substrate to the $NADP^+$ dependent isocitrate dehydrogenase isoenzyme [23] to produce NADPH. Acetoacetate and β-hydroxybutyrate are natural fuels which elevate myocardial citrate content and may act as antioxidants by pyruvate-like mechanisms. This study tested the hypothesis that these ketone bodies increase GSH redox potential and contractile performance of H_2O_2-injured myocardium.

METHODS

Isolated working heart model. All animal experimentation was approved by the Animal Care and Use Committee of the University of North Texas Health Science Center. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Hearts excised from male Hartley guinea pigs (n= 43; 575 ± 22 g) were antegradely perfused in the working configuration. All Krebs-Henseleit perfusion media [24] were maintained at 38°C, aerated with 95% O_2: 5% CO_2, and fortified with 10 mM glucose. Heart rate, aortic pressure (P_a), left atrial filling pressure (P_v), and cardiac output (the sum of aortic and coronary flows) were measured to assess cardiac function. Stroke work was computed from stroke volume and left ventricular developed pressure, i.e. P_a-P_v. Cardiac power was expressed as the product of stroke work and heart rate.

Hydrogen peroxide injury protocol. Following 15 min perfusion with standard Krebs-Henseleit medium, the hearts were challenged by 10 min perfusion with medium containing 100 μM H_2O_2. Next, hearts were perfused for 30 min with H_2O_2-free medium, then for 60 min with media containing 5 mM acetoacetate or β-hydroxybutyrate. Hearts were then freeze-clamped with liquid N_2 cooled Wollenberger tongs and stored at –90°C. Cardiac extracellular space was taken as sucrose distribution volume, determined by continuously infusing sucrose to a left atrial concentration of 2 M for 5 min prior to freeze-clamp [24]. Intracellular volume equaled tissue water content (ml/g) minus extracellular space.

Myocardial metabolites. Frozen hearts were pulverized under liquid N_2, then extracted [25] for measurement of ATP, phosphocreatine (PCr), creatine (Cr), inorganic phosphate (P_i), citrate, glucose 6-phosphate and sucrose by standard spectrophotometric assays [26]. Intracellular P_i concentration was computed as [(total myocardial P_i content – extracellular P_i content) ÷ intracellular volume (ml/g)], where extracellular P_i content equaled [sucrose distribution volume × ((perfusion medium P_i concentration + coronary effluent P_i concentration)/2)]. Phosphocreatine phosphorylation potential ([PCr]/([Cr][P_i])), an indicator of ATP phosphorylation potential via the near-equilibrium creatine kinase reaction [27], was calculated as an index of myocardial energy state [24]. Glutathione (GSH) and glutathione disulfide (GSSG) were measured by the procedures of Akerboom and Sies [28] as recently described [18]. GSH redox potential ({GSH}/{GSSG}) was taken as an index of the global redox state of the myocardial antioxidant defenses [29]. Metabolites were determined in a Shimadzu model UV-1601PC dual wavelength uv/vis spectrophotometer (337 nm measuring wavelength, 417 nm reference wavelength, $\varepsilon = 5.65\ M^{-1} \cdot cm^{-1}$).

Acetoacetate-H_2O_2 reaction assay. Experiments were conducted *in vitro* to determine whether acetoacetate directly neutralizes H_2O_2 in a nonenzymatic reaction similar to pyruvate's H_2O_2-detoxifying mechanism [19]. H_2O_2 (65 μM) was added to 70 mM phosphate-buffered (pH 6.0) solutions containing 0-2 mM acetoacetate or pyruvate. After 60 min incubation, H_2O_2 was spectrophotometrically assayed at 420 nm in the

presence of horseradish peroxidase and 2,2'-azino-di-[3-ethyl-benzothiazoline-(6)-sulphonic acid [26].

Statistical Analyses. Data are presented as means ± SEM. Within-group effects of acetoacetate or β–hydroxybutyrate treatment on cardiac performance were analyzed by comparing pre-treatment values measured at 28-30 min post-H_2O_2 with values recorded at 60 min treatment using paired Student's t-test. Coronary flows and resistances measured at 10 min H_2O_2 *vs.* 90 min post-H_2O_2 in untreated hearts were compared by paired Student's t-test. Cardiac power, coronary hemodynamics and metabolite data in the different groups were compared by one-way ANOVA. When ANOVA detected significant differences, Student-Newman-Keul's multiple comparison test was applied to detect the specific differences. Statistical significance was assumed at $P < 0.05$.

RESULTS

Contractile performance of hydrogen peroxide-injured myocardium. Cardiac power fell steeply during 10 min H_2O_2 exposure, and continued to decline during the first 15 min H_2O_2 washout before stabilizing at 30% of pre-H_2O_2 baseline (Figure 1). Cardiac power in H_2O_2-free time control hearts fell by less than 20% over the 2 h protocol, indicating adequate functional stability of the isolated heart preparation.

Acetoacetate administered 30-90 min post-H_2O_2 nearly restored cardiac power to the time control level (Figure 1). This enhancement of contractile performance was fully manifest by 15 min treatment and was sustained for the remainder of the treatment period. In contrast, β-hydroxybutyrate, the product of acetoacetate reduction via the near-equilibrium β-hydroxybutyrate dehydrogenase reaction, failed to improve cardiac function.

Coronary flow and resistance. Despite the decline in power during 10 min H_2O_2 exposure, coronary flow increased 60% (Figure 2) due to a 59% drop in coronary resistance. In untreated hearts, coronary flow and resistance recovered by 90 min H_2O_2-washout. Neither acetoacetate nor β-hydroxybutyrate altered flow or resistance *vs.* the untreated group. Therefore, acetoacetate's tendency to increase coronary flow in H_2O_2-injured hearts (Figure 2) resulted from increased P_a, *i.e.* coronary perfusion pressure, not from coronary vasodilation.

Myocardial energy metabolites. Myocardial ATP content and phosphocreatine (PCr) phosphorylation potential, *i.e.* [PCr]/([Cr][P_i]), were measured to determine myocardial energy state. ATP, PCr, and creatine (Cr) contents tended to be lower in all three groups of H_2O_2-challenged hearts than in H_2O_2-free time controls; indeed, these depletions were statistically significant in the acetoacetate group (Figure 3A). Neither acetoacetate nor β-hydroxybutyrate altered ATP, PCr, or Cr content of post-H_2O_2 myocardium. PCr phosphorylation potential of untreated myocardium at 90 min post-H_2O_2 was similar to the time control value. Although acetoacetate and β-hydroxybutyrate tended to moderately increase phosphorylation potential (Figure 3B), these changes were not statistically significant.

Glutathione redox state. Myocardial glutathione (GSH) content was measured to assess the capacity of the glutathione antioxidant system, and glutathione/glutathione disulfide (GSH/GSSG) redox state was determined as an index of myocardial redox potential. 10 min H_2O_2 exposure and 90 min washout depleted GSH content by 40% (Figure 4A), indicating that prooxidant exposure depleted myocardial antioxidant capacity. Neither acetoacetate nor β-hydroxybutyrate increased GSH content but both treatments lowered GSSG *vs.* untreated and time control hearts, acetoacetate to a greater extent than β-hydroxybutyrate. GSH/GSSG redox potential fell 46% after H_2O_2 challenge and washout (Figure 4B). Acetoacetate powerfully increased GSH redox potential by 140% (Figure 4B). β-hydroxybutyrate also increased GSH/GSSG, but only by 60%. Thus, both metabolic

treatments bolstered GSH redox potential depleted by H_2O_2 exposure, but to different extents.

Myocardial citrate and glucose 6-phosphate. Glutathione is regenerated from GSSG by glutathione reductase using reducing equivalents supplied by NADPH. Citrate could increase NADPH and thus GSH redox potential by undergoing conversion to isocitrate, thereby providing substrate for $NADP^+$-dependent isocitrate dehydrogenase. Alternatively, citrate inhibits phosphofructokinase and would divert glucose 6-phosphate into the NADPH-generating hexose monophosphate shunt. In accordance with these mechanisms, both metabolic treatments increased citrate severalfold and glucose 6-phosphate content by approximately 50% in H_2O_2-injured myocardium (Figure 5).

Direct detoxification of H_2O_2. by acetoacetate. Pyruvate and other α-keto carboxylates detoxify H_2O_2 in a direct, nonenzymatic reaction [19]. Experiments were conducted *in vitro* to determine whether acetoacetate consumed H_2O_2 by a similar process. H_2O_2 was added to solutions containing 0-2 mM acetoacetate or pyruvate, then measured after 60 min. As expected, pyruvate concentration-dependently depleted H_2O_2; 2 mM pyruvate completely eliminated the prooxidant. On the other hand, acetoacetate did not consume H_2O_2 at any concentration (Figure 6). Thus, direct H_2O_2 detoxification probably did not contribute to acetoacetate's antioxidant properties in myocardium.

DISCUSSION

This study was conducted to determine the ability of acetoacetate and β-hydroxybutyrate to augment contractile performance and antioxidant redox potential in H_2O_2-challenged myocardium. H_2O_2 impaired contractile function, depleted GSH reserves and lowered GSH redox potential, the major endogenous antioxidant defense system in myocardium and a measure of the global antioxidant redox state [29]. Acetoacetate nearly restored cardiac power, but its reduced congener β-hydroxybutyrate failed to improve function of H_2O_2-impaired myocardium. Although both compounds are oxidizable fuels in myocardium, neither increased phosphocreatine phosphorylation potential, a measure of cellular energy state [27]. Neither ketone body increased GSH content, but both increased glutathione redox potential *vs.* untreated post-H_2O_2 myocardium. Acetoacetate's enhancement of GSH/GSSG was considerably more robust than β-hydroxybutyrate's. Apparently, acetoacetate restored cardiac function post-H_2O_2 by antioxidant but not energy-linked mechanisms.

Peroxide-induced myocardial injury. Several mechanisms have been proposed for H_2O_2 induction of cardiac injury and contractile dysfunction. H_2O_2 causes cytosolic calcium overload [30], sarcolemmal disruption [31], impairment of glycolytic [4, 7, 8] and oxidative metabolism [4, 8, 11-13], and depletion of endogenous antioxidant defenses [32, 33]. Severe contractile impairment of the isolated working guinea-pig hearts, accompanied by GSH depletion and a persistent, albeit partial, decrease of GSH/GSSG redox potential are indicative of oxidative stress in this H_2O_2 injury model. We recently demonstrated that 10

min H_2O_2 exposure decreased phosphocreatine phosphorylation potential 35% [17], but phosphorylation potential recovered to the time control level by 90 min post-H_2O_2. Decreased energy demand for contractile work in these weakened hearts may have restored the balance between myocardial energy demand and decreased energy production, allowing energy reserves to recover.

Ketone bodies and the cardiac glutathione system. In this investigation, hearts were subjected to a H_2O_2 perfusion-washout protocol that depleted myocardial GSH content by approximately 33% and lowered the GSH/GSSG ratio to 50% of the time control value. Acetoacetate and to a lesser extent β-hydroxybutyrate lowered GSSG and, thus, increased GSH/GSSG *vs.* untreated H_2O_2-challenged hearts, although GSH content remained depleted. The glutathione system is the central component of the myocardium's intrinsic antioxidant defenses [32], and the GSH/GSSG ratio provides a global index of oxidative damage and antioxidant redox state within tissue [29]. Thus the ketone bodies, especially acetoacetate, augmented myocardial antioxidant redox potential that had been diminished by H_2O_2.

Acetoacetate did not directly neutralize H_2O_2 unlike the α–keto carboxylate pyruvate. However, acetoacetate and β-hydroxybutyrate may act indirectly as antioxidants by generating citrate. An inhibitor of phosphofructokinase [22], citrate could divert glycolytic flux into the hexose monophosphate shunt. This pathway generates NADPH, the source of reducing equivalents to maintain GSH reducing power [14]. Also, the

conversion of citrate to isocitrate would support NADPH formation by providing substrate to the $NADP^+$-dependent isocitrate dehydrogenase [23].

In addition to the citrate-mediated mechanisms, acetoacetate or its thioester derivative acetoacetyl CoA could detoxify prooxidants by more direct mechanisms. The cytotoxic prooxidant peroxynitrite ($ONOO^-$) is formed when nitric oxide irreversibly condenses with superoxide ($\cdot O_2^-$) generated from H_2O_2 by reversal of the superoxide dismutase reaction [34]. Acetoacetate consumes $ONOO^-$ in an aliphatic nitration reaction that generates nontoxic products [35]. Conversion of acetoacetate to acetoacetyl-CoA by 3-oxoacid-CoA transferase may also attenuate H_2O_2-injury. Acetoacetyl-CoA, which is elevated in myocardium perfused with excess acetoacetate [36], directly consumes H_2O_2 to generate an unidentified free radical intermediate which is subsequently neutralized by electrons supplied by NADH [37]. The ketone bodies would mitigate the H_2O_2-imposed burden on the glutathione system by lessening oxidative stress.

Although acetoacetate and β-hydroxybutyrate increased myocardial citrate content to similar extents, β-hydroxybutyrate only tended to increase GSH/GSSG redox state and failed to augment contractile function. β-hydroxybutyrate's ineffectiveness as an antioxidant and cardiotonic treatment underscores the importance of acetoacetate's citrate-independent antioxidant mechanisms. Unlike acetoacetate, β-hydroxybutyrate does not directly neutralize $ONOO^-$. Moreover, β-hydroxybutyrate would not increase intracellular acetoacetate or acetoacetyl-CoA as effectively as exogenous acetoacetate,

due to the limitation imposed by the poise of the β-hydroxybutyrate dehydrogenase equilibrium.

Effects of acetoacetate vs. *β-hydroxybutyrate on cardiac function and phosphorylation potential.* Acetoacetate increased contractile performance of H_2O_2 injured hearts to near that of uninjured time controls without increasing myocardial phosphorylation potential, a measure of the energy reserve available to support contractile activity [27]. Moreover, phosphorylation potential was nearly identical in β-hydroxybutyrate treated myocardium but function in those hearts was well below that of the acetoacetate treated hearts. Thus, acetoacetate's contractile effects could not be ascribed to enhancement of cellular energy state. Instead, acetoacetate's enhancement of myocardial contractile performance and, thus, energy demand may have prevented myocardial phosphorylation potential from increasing.

Total creatine content ($\mu mol \cdot g\ dry^{-1}$) fell in the three H_2O_2-challenged groups (untreated: 63 ± 3; acetoacetate treated: 51 ± 3; β-hydroxybutyrate treated: 53 ± 4) *vs.* time controls (77 ± 6). Creatine may have been consumed in the direct neutralization of H_2O_2 and its cytotoxic metabolites [38]. If such creatine-consuming antioxidant reactions did indeed occur, they did not prevent contractile dysfunction following H_2O_2 exposure.

Clinical implications. Acetoacetate could function as an antioxidant in clinical situations in which its circulating concentration is increased. Plasma ketone bodies are elevated in severe, poorly controlled diabetes mellitus, a condition that inflicts oxidative injury in

tissues. If the current findings in myocardium are generally applicable to other tissues, acetoacetate might serendipitously alleviate the prooxidant burden of diabetes and slow progression of the disease. However, acetoacetate's citrate-mediated antioxidant mechanisms would require glucose metabolism, which is impaired in diabetes. Although the role of glucose in acetoacetate's salutary mechanisms has not been determined, glucose metabolism would be required to provide substrate for the hexose monophosphate pathway and pyruvate for anaplerotic carboxylation [21].

Limitations. Metabolites were measured in extracts of flash-frozen myocardium, which precluded assessment of subcellular metabolite compartmentation in mitochondria and other organelles. Mitochondria contain approximately 15% of the total glutathione pool in rat heart [39]. Despite indications that cytosolic and mitochondrial GSH/GSSG redox potentials equilibrate [40], the mitochondrial and cytosolic redox environments could potentially differ in H_2O_2-challenged and ketone body treated myocardium. It has been demonstrated that acetoacetate as the lone oxidizable fuel cannot maintain cardiac function due to its inability to provide anaplerotic substrate to the TCA cycle [21]. Accordingly, the current study combined the ketone body treatments with 10 mM glucose as the basal energy-yielding fuel. Whether acetoacetate's antioxidant character requires glucose as cosubstrate is unclear.

Although this study demonstrates for the first time the inotropic and antioxidant actions of acetoacetate in prooxidant-challenged myocardium, the mechanisms by which

acetoacetate restores contractile performance are unclear. The specific cellular components protected by acetoacetate remain to be identified but likely include protein or phospholipid targets of H_2O_2 and its metabolites [41]. Furthermore, H_2O_2 is but one of several reactive oxygen species implicated in ischemia/reperfusion injury. Whether acetoacetate exerts salutary effects in this more complex situation remains to be determined.

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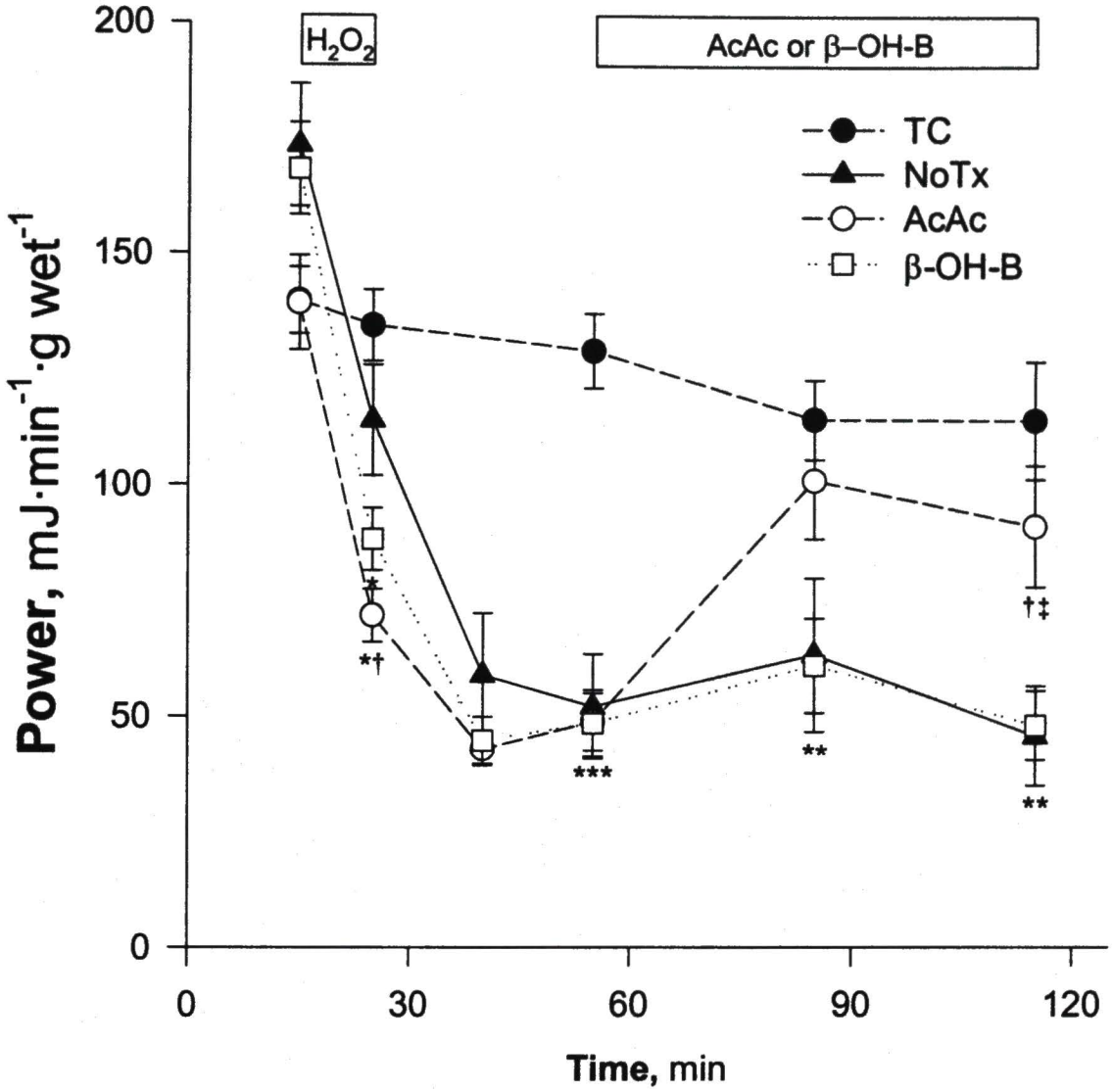

Figure 1. *Effects of H_2O_2 exposure and ketone bodies on cardiac power.* Values are means ± SEM. Bars at the top of the figure indicate the periods of H_2O_2 perfusion and ketone body treatment. TC: time control (n=14); NoTx: untreated H_2O_2-challenged (n=9); AcAc: acetoacetate treated (n=9); β-OH-B: β-hydroxybutyrate treated (n=7). *P<0.05 *vs.* TC; †P<0.05 *vs.* NoTx; ‡P<0.05 *vs.* (pre-treatment baseline) 55 min.

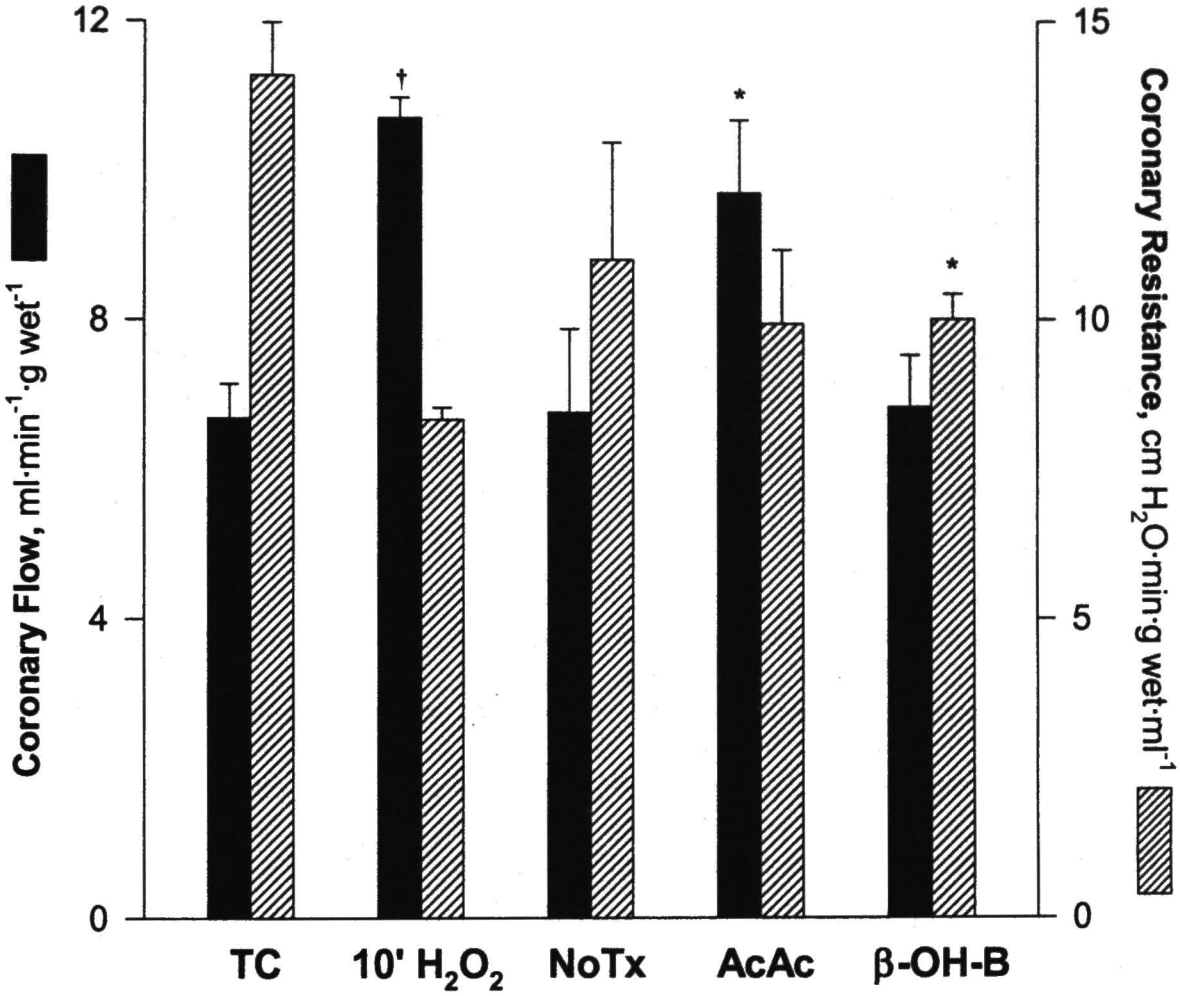

Figure 2. *Impact of H_2O_2-challenge and ketone bodies on coronary hemodynamics.* Coronary flow and resistance were measured at 10 min H_2O_2 perfusion (10' H_2O_2) and at 115 min perfusion. Abbreviations as in Figure 1. *P<0.05 *vs.* TC; †P<0.05 *vs.* NoTx.

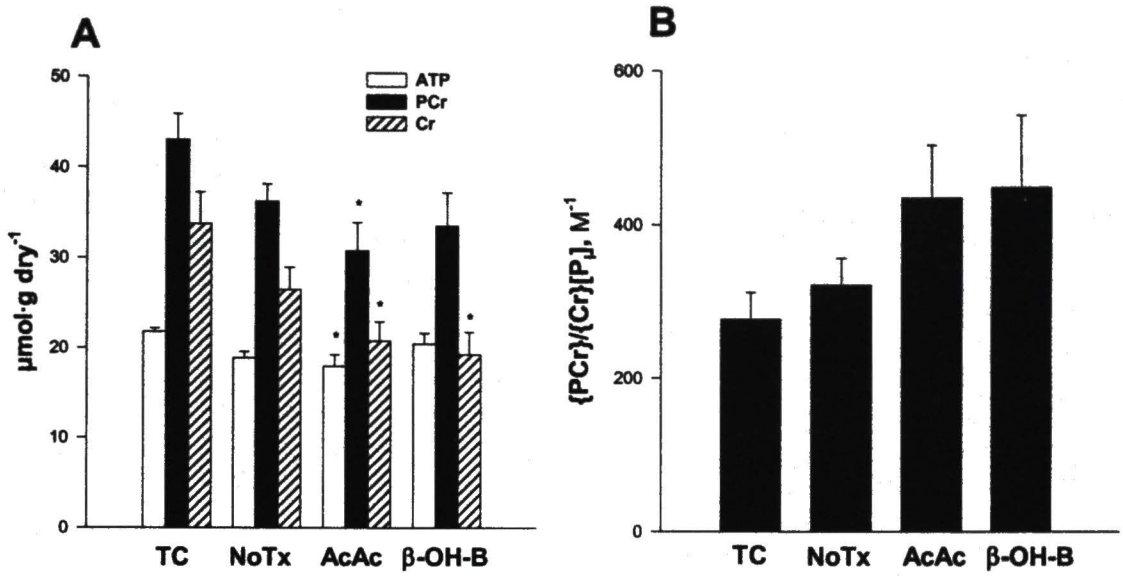

Figure 3. *Myocardial energy metabolites and phosphorylation potential.* Panel A: Metabolites (PCr: phosphocreatine, Cr: creatine) were measured in myocardium snap-frozen at 115 min perfusion. Panel B: Phosphocreatine phosphorylation potential was calculated from PCr and Cr contents ({}) and intracellular inorganic phosphate concentration ($[P_i]$). Abbreviations as in Figure 1. *$P<0.05$ *vs.* TC.

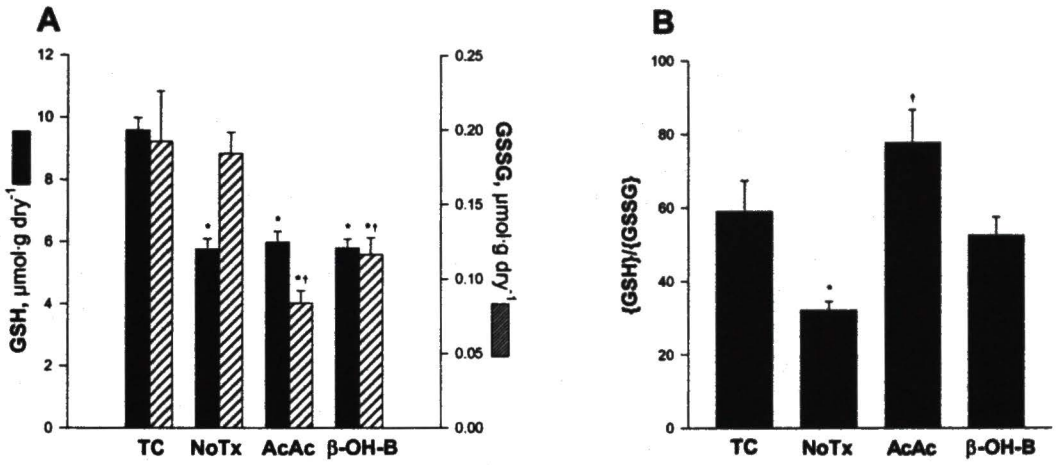

Figure 4. *Glutathione redox state.* Glutathione (GSH) and glutathione disulfide (GSSG) contents (Panel A), measured in snap-frozen myocardium, were used to calculate glutathione redox potential, *i.e.* {GSH}/{GSSG}; (Panel B). Abbreviations as in Figure 1. *P<0.05 *vs.* TC; †P<0.05 *vs.* NoTx.

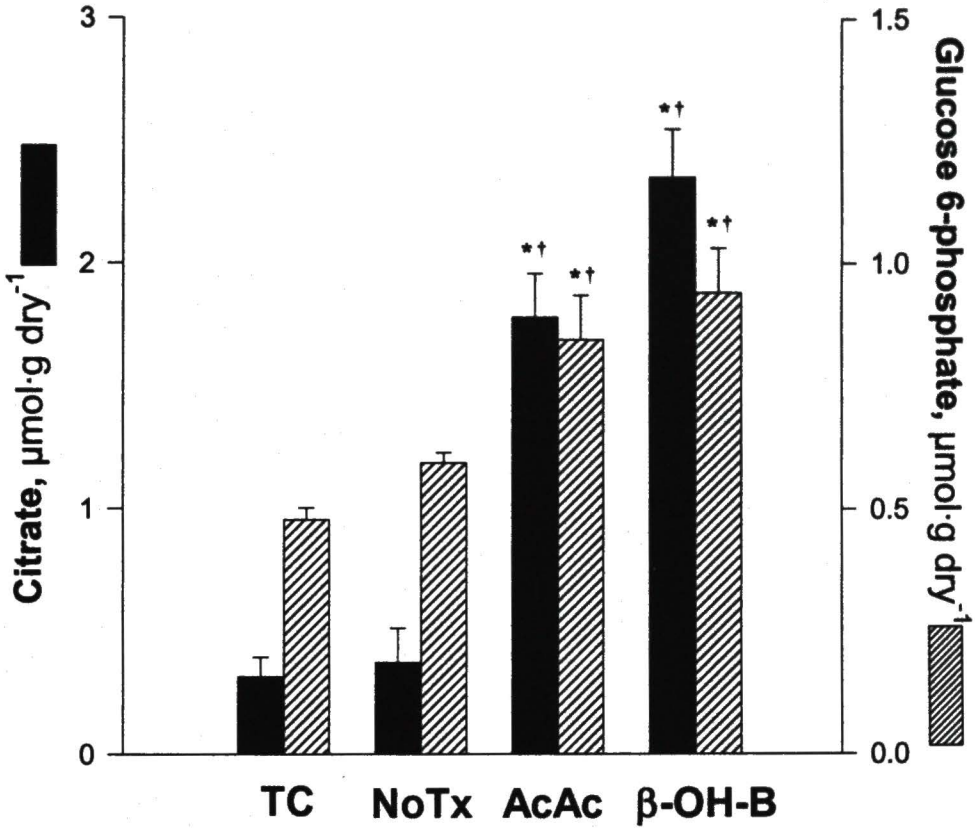

Figure 5. *Myocardial citrate and glucose 6-phosphate.* Citrate and glucose 6-phosphate contents were measured in the same hearts as in Figures 1-4. *P<0.05 *vs.* TC; †P<0.05 *vs.* NoTx.

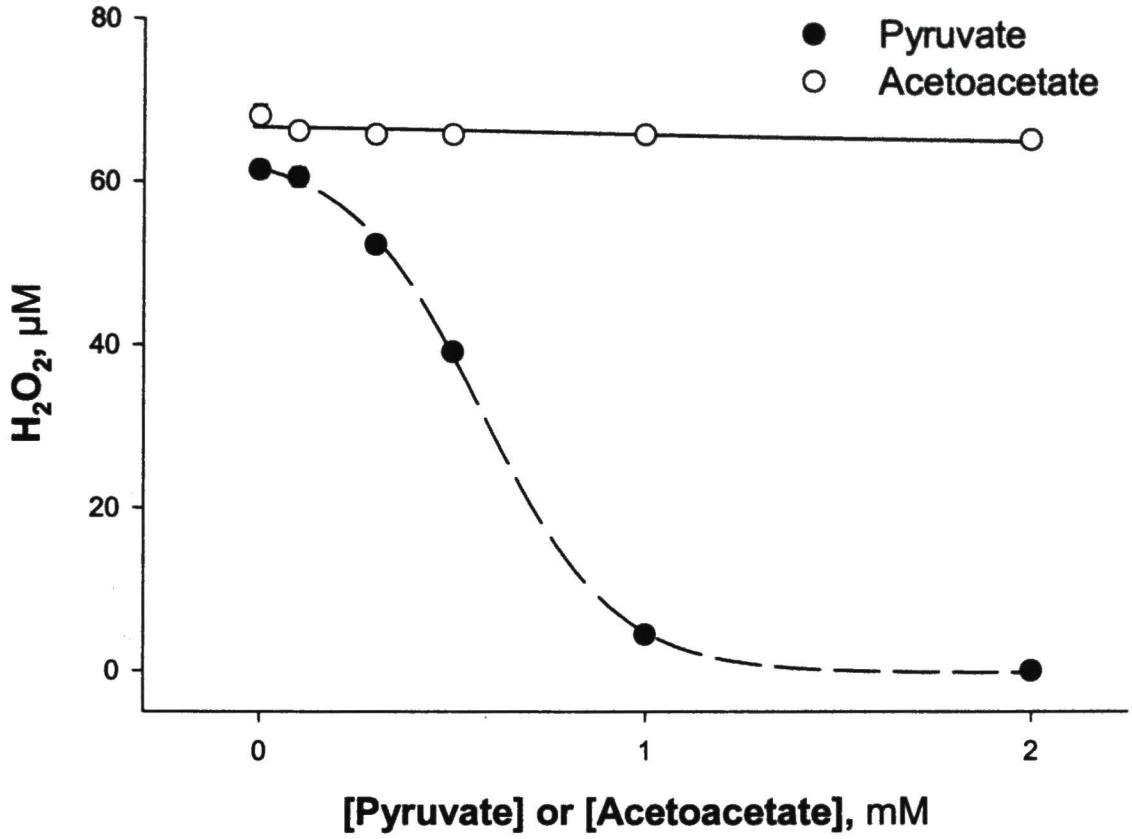

Figure 6. *Direct H_2O_2 detoxification by pyruvate* vs. *acetoacetate.* H_2O_2 concentration was measured following 60 min incubation in phosphate buffered solutions containing 0-2 mM acetoacetate (open circles) or pyruvate (filled circles). Values are means ± SEM from 3 measurements.

CHAPTER III

Acetoacetate Augments β–Adrenergic Inotropism of Stunned Myocardium by an Antioxidant Mechanism

Jeffrey E. Squires, Jie Sun, James L. Caffrey, Darice Yoshishige, and Robert T. Mallet

ABSTRACT

Blunted β–adrenergic inotropism in stunned myocardium is restored by pharmacological (*N*-acetylcysteine) and metabolic (pyruvate) antioxidants. The ketone body acetoacetate is a natural myocardial fuel and antioxidant that improves contractile function of prooxidant-injured myocardium. The impact of acetoacetate on post-ischemic cardiac function and β–adrenergic signaling has never been reported. To test the hypothesis that acetoacetate restores contractile performance and β–adrenergic inotropism of stunned myocardium, post-ischemic Krebs-Henseleit perfused guinea-pig hearts were treated with 5 mM acetoacetate and/or 2 nM isoproterenol at 15-45 and 30-45 min reperfusion, respectively, while cardiac power was monitored. The myocardium was snap-frozen and its energy state was assessed from phosphocreatine phosphorylation potential. Antioxidant defenses were assessed from GSH/GSSG and $NADPH/NADP^+$ redox potentials. Stunning lowered cardiac power and GSH redox potential by 90% and 70%, respectively. Given separately, acetoacetate and isoproterenol each increased power and GSH redox potential three-to-fourfold. Phosphocreatine potential was 70% higher in acetoacetate *vs.* isoproterenol treated hearts ($P<0.01$). In combination, acetoacetate and isoproterenol synergistically increased power and GSH redox potential 16- and 7-fold, respectively, doubled NADPH redox potential and increased cyclic AMP content 30%. The combination increased cardiac power 6-8 fold *vs.* the individual treatments without a coincident increase in phosphorylation potential. Thus, acetoacetate increased contractile performance and potentiated β–adrenergic inotropism in stunned myocardium without

increasing energy reserves, suggesting its antioxidant character is central to its beneficial actions.

Abbreviations: AcAc: acetoacetate; cAMP: cyclic AMP; Cr: creatine; ISO: isoproterenol; NoTx: no treatment; P_a: aortic pressure; PCr: phosphocreatine; P_v: left atrial filling pressure; TC: time control

INTRODUCTION

Post-ischemic myocardial stunning is produced in large part by cytotoxic oxygen and nitrogen metabolites generated upon coronary reperfusion (5). The resulting superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and peroxynitrite ($ONOO^-$) (2, 12, 34), modify a variety of cellular components (6), to produce stunning. Inotropic responses to β–adrenergic stimulation are dampened in stunned myocardium (33, 39), perhaps due to oxidative damage to protein components of the β–adrenergic signaling cascade.

Pyruvate, a natural metabolic fuel in myocardium, markedly increased β–adrenergic responsiveness of stunned myocardium and preserved energy stores (39). Pyruvate also restored GSH/GSSG redox potential (38), the principal intracellular antioxidant system, and bolstered $NADPH/NADP^+$ redox potential, the source of reducing power to regenerate GSH from GSSG (26). *N*-acetylcysteine, a membrane permeable antioxidant, recapitulated the pyruvate enhancement of β–adrenergic inotropism despite the failure of this non-fuel to prevent β–adrenergic depletion of myocardial energy reserves. Like pyruvate, *N*-acetylcysteine increased GSH redox potential. These combined findings indicated that pyruvate's antioxidant actions, more than its enhancement of energy reserves, mediated its restoration of β–adrenergic inotropism in stunned myocardium (38).

We recently reported that acetoacetate sharply increased GSH redox potential and contractile performance of myocardium challenged by H_2O_2 in the absence of ischemia (37). If acetoacetate exerts similar antioxidant actions in stunned myocardium, then it could potentiate β–adrenergic inotropism as did pyruvate and *N*-acetylcysteine. To test this proposal, post-ischemic, stunned guinea-pig hearts were treated with 5 mM acetoacetate alone or in combination with isoproterenol at a low concentration (2 nM) that only modestly increases contractile performance in the absence of antioxidants (38, 39). The impact of these treatments on left ventricular contractile performance was compared with their effects on myocardial energy reserves, antioxidant redox potential, and cyclic AMP. This study demonstrated that acetoacetate only modestly increased contractile performance of stunned myocardium, but powerfully potentiated inotropic responses to isoproterenol. These contractile responses paralleled acetoacetate's augmentation of GSH redox potential, which was potentiated by isoproterenol coadministration.

MATERIALS AND METHODS

Isolated working hearts. Animal experimentation was approved by the Animal Care and Use Committee of the University of North Texas Health Science Center and was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, USA, 1996). Hearts (n = 47) were excised from male Hartley guinea pigs (400g–600g) and antegradely perfused as working organs with Krebs-Henseleit bicarbonate buffer (39). All perfusion media were maintained at 38°C, aerated with 95% O_2 : 5% CO_2, and fortified with 10 mM glucose. Heart rate, aortic pressure (P_a), left atrial filling pressure (P_v), and cardiac output (sum of aortic and coronary flows) were measured to determine cardiac function. Left ventricular function was assessed from stroke work ($mJ \cdot g^{-1}$) and power ($mJ \cdot min^{-1} \cdot g^{-1}$), which equaled stroke work times heart rate.

Ischemia-reperfusion protocol to produce cardiac stunning. Following 15 min of preischemic baseline perfusion, hearts were subjected to 45 min coronary underperfusion by lowering P_a, and concomitantly stimulated with 0.4 μM *L*-norepinephrine (39). Ischemic hearts were reperfused by restoring P_a to 90 cm H_2O and discontinuing *L*-norepinephrine infusion. P_a subsequently declined before stabilizing by 10-15 min reperfusion at 40-50 cm H_2O, reflecting contractile impairment typical of myocardial stunning (39). P_v was held at 10-12 cm H_2O throughout the protocol.

Metabolic and β–adrenergic treatment of stunned hearts. Four groups of stunned hearts were examined: no treatment (n = 12), 5 mM acetoacetate at 15-45 min reperfusion (n = 6), 2 nM isoproterenol at 30-45 min reperfusion (n = 8), or combined treatment with 5 mM acetoacetate at 15-45 min and 2 nM isoproterenol at 30-45 min (n = 9). Contractile performance and metabolic state of these stunned hearts were compared with time control hearts (n = 12) perfused for 105 min without ischemia or treatment. Isoproterenol stock solution (100 nM) was freshly prepared 10-15 min before infusion in 0.9% NaCl/1% ascorbic acid, and shielded from light to prevent auto-oxidation. Sucrose was continuously infused to achieve a 2 mM left atrial concentration during the final 5 min to determine extracellular space as recently described (39). All hearts were freeze-clamped with liquid N_2- precooled Wollenberger tongs and stored at -90°C before metabolite extraction.

Myocardial Metabolites. Frozen hearts were pulverized in a precooled porcelain mortar under liquid nitrogen. The powdered tissue was extracted (16, 24) for measurement of ATP, phosphocreatine (PCr), creatine (Cr), P_i, citrate, glucose 6-phosphate, $NADP^+$, NADPH, GSH, GSSG, and sucrose. Metabolites were assayed (4) in a Shimadzu model UV-1601PC dual wavelength uv/vis spectrophotometer (337 nm measuring wavelength, 417 nm reference wavelength, $\varepsilon = 5.65\ M^{-1}\cdot cm^{-1}$). Intracellular P_i was determined by subtracting extracellular P_i, *i.e.* perfusate P_i concentration times sucrose distribution volume, from total myocardial P_i content (24, 30). Phosphocreatine phosphorylation potential ([PCr]/([Cr][P_i])) was calculated as an index of cellular energy state (39). GSH

and GSSG were measured according to Akerboom and Sies (1). Myocardial cyclic AMP content was determined by radioimmunoassay (25) as recently described (39).

Statistical Analyses. Data are expressed as means ± SEM. Cardiac performance was analyzed using two-way ANOVA. Metabolites, phosphorylation potential, and GSH and NADPH redox states in the different groups were compared by one-way ANOVA. Within-group comparisons at different times in the experiment were accomplished with one-way ANOVA for repeated measures. When ANOVA detected significant differences, Student-Newman-Keuls multiple comparison test was used *post hoc* to identify the specific differences. Statistical significance was assumed at $P < 0.05$.

RESULTS

Cardiac function. Contractile responses to 2 nM isoproterenol and/or 5 mM acetoacetate were determined in post-ischemic hearts. In stunned non-treated hearts, cardiac power stabilized at approximately 10% of the nonischemic time control value (Figure 1) indicating severe contractile impairment of post-ischemic myocardium. Acetoacetate or isoproterenol alone increased power three- and fourfold, respectively, *vs.* pretreatment baseline. Combined, acetoacetate + isoproterenol treatment elicited a much more robust response that far exceeded the sum of the individual treatment effects: here, cardiac power increased 16-fold, to 156% of the time control value. Thus, the combined metabolic and β-adrenergic treatments elicited a powerful, synergistic enhancement of cardiac performance.

Cyclic AMP. The intracellular second messenger for β–adrenergic signaling, cyclic AMP was measured to determine the impact of stunning and post-ischemic treatments on the signaling mechanism. Neither stunning *per se* nor treatment of stunned myocardium with 2 nM isoproterenol altered cyclic AMP content *vs.* time control myocardium (Figure 2). Treatment with acetoacetate alone unexpectedly depleted cyclic AMP by 30% versus untreated or isoproterenol-treated stunned myocardium. In contrast, treatment with acetoacetate in combination with isoproterenol increased cyclic AMP content significantly above all other groups. Indeed, the combined treatments increased cyclic AMP content by 30% over treatment with isoproterenol alone, indicating acetoacetate potentiated β–adrenergic signaling.

Myocardial phosphorylation potential. Myocardial energy state was assessed from phosphocreatine phosphorylation potential ([PCr]/([Cr][P_i])), a measure of cytosolic ATP phosphorylation potential (41). Phosphocreatine potential of the untreated stunned myocardium was similar to the time control value (Figure 3), despite the disparity in contractile performance of these two groups. Acetoacetate tended to increase, and isoproterenol to lower, phosphorylation potential *vs.* stunned myocardium, although neither effect was significant. Moreover, phosphorylation potential of the acetoacetate hearts was 70% greater than that of isoproterenol-treated hearts ($P<0.01$) at similar levels of mechanical performance (Figure 1). Combining acetoacetate with isoproterenol prevented further decline in myocardial energy state (Figure 3) despite the severalfold increase in contractile function (Figure 1).

Glutathione redox potential. Components of the GSH:NADPH redox system were measured to determine the impact of metabolic and inotropic treatments on antioxidant redox potential of stunned myocardium. GSH content tended to be lower in untreated stunned *vs.* time control myocardium, but the difference was not statistically significant. GSSG doubled, and the GSH/GSSG ratio, a measure of cellular antioxidant redox potential (35), fell 71% in stunned *vs.* time control myocardium (Figure 4). Treatment with acetoacetate alone lowered GSSG content 68% and quadrupled GSH/GSSG redox potential in stunned myocardium. Isoproterenol also lowered GSSG, although not as much as acetoacetate, and tripled GSH/GSSG. Remarkably, when combined, the two

interventions powerfully increased GSH/GSSG redox state to double the time control value. Myocardial GSH content was similar among the treatments, so GSH/GSSG redox potential was altered in each case by changes in GSSG content alone.

GSH redox potential is maintained by glutathione reductase, which transfers reducing equivalents supplied by NADPH to GSSG to regenerate GSH. Thus, the NADPH/$NADP^+$ redox system provides the reducing power to maintain GSH/GSSG. Neither NADPH nor $NADP^+$ content, nor the NADPH/$NADP^+$ ratio were altered by stunning (Figure 5). Acetoacetate lowered $NADP^+$ content and increased NADPH/$NADP^+$ ratio by 63%. Isoproterenol alone did not alter NADPH redox state. In contrast, combined isoproterenol + acetoacetate decreased $NADP^+$ content by 47% and doubled the NADPH/$NADP^+$ ratio. Thus, acetoacetate, alone and especially in combination with isoproterenol, increased NADPH reducing power to bolster GSH redox potential in stunned myocardium.

Citrate and glucose-6-phosphate. NADPH is generated in myocardium by two metabolic mechanisms mediated by citrate. Citrate accumulation could promote flux through the cytosolic, $NADP^+$-specific isocitrate dehydrogenase reaction by providing substrate for aconitase-catalyzed isocitrate formation. Secondly, citrate could increase NADPH formation by inhibiting phosphofructokinase (17), which would divert glycolytic flux into the NADPH-generating hexose monophosphate shunt. In the latter scenario, glucose 6-phosphate, a glycolytic intermediate proximal to phosphofructokinase, would

accumulate and provide substrate for the hexose monophosphate pathway. To test the possibility that acetoacetate and/or isoproterenol activated NADPH-generating pathways, citrate and glucose 6-phosphate were measured in stunned and time control hearts. Contents of these compounds did not differ in stunned *vs.* time control myocardium (Figure 6). Given seperately, acetoacetate and isoproterenol tended to double citrate content and increase glucose 6-phosphate content by roughly 35-45%, but only the acetoacetate induced increase in glucose 6-phosphate was statistically significant *vs.* untreated stunned myocardium. In combination, the two treatments increased citrate fourfold and doubled glucose 6-phosphate content *vs.* time control and untreated stunned myocardium. Thus, acetoacetate in combination with isoproterenol powerfully increased myocardial citrate and glucose 6-phosphate contents, bolstering substrate supply for pathways generating NADPH reducing power.

DISCUSSION

Recently pyruvate's antioxidant properties were shown to potentiate β–adrenergic inotropism and cyclic AMP formation in post-ischemic stunned myocardium (39). Pyruvate appeared to enhance β–adrenergic inotropism by augmenting GSH and NADPH antioxidant redox potentials (38). This antioxidant effect may have restored the redox status of proteins in the β–adrenergic signaling cascade. The ketone body acetoacetate, a natural energy-yielding fuel in myocardium, increased GSH/GSSG and contractile performance of hydrogen peroxide injured myocardium (37). Thus acetoacetate, like pyruvate, might increase contractile performance and potentiate β–adrenergic inotropism in stunned myocardium by antioxidant mechanisms.

Acetoacetate increased contractile function of the stunned myocardium. Although modest, this enhanced function was similar to that observed with the same concentration of pyruvate in the post-ischemic myocardium (39). Concentrations of isoprotetenol that produced a modest increase in contractile performance were dramatically enhanced following pretreatment with acetoacetate. Indeed the combination of acetoacetate and isoproterenol produced a six to eight-fold increase in cardiac power compared to either acetoacetate or isoproterenol alone. Thus acetoacetate, like pyruvate (39), dramatically improved β–adrenergic inotropism in the stunned myocardium.

Glutathione redox potential and cyclic AMP formation. The GSH system is the central component of the myocardium's endogenous defenses against oxidative damage (36).

GSH neutralizes peroxides (13) and peroxynitrite (12) to prevent oxidative damage. GSH also restores oxidized protein sulfhydryls to their reduced state (15) and thereby maintains the catalytic activity of enzymes susceptible to oxidative stress (11, 32). Myocardial stunning reduces the heart's sensitivity to β-adrenergic stimulation by lowering the binding affinity and density of β_1-adrenergic receptors and by inactivating adenylate cyclase (33) through the oxidation of sulfhydryl groups within the enzyme (14). Glutathione redox potential fell by 71% in stunned myocardium indicating appreciable oxidative stress due to ischemia/reperfusion. Isoproterenol increased GSH redox potential modestly and acetoacetate restored GSH potential to the time control level. Once again, the combined interventions increased GSH redox potential well above either agent alone. By augmenting the reducing power of the GSH system, acetoacetate may have helped restore sulfhydryls of proteins inactivated by oxidative stress, including β-adrenergic signaling proteins.

Cyclic AMP was measured to establish the impact of metabolic and inotropic treatments on post-ischemic β-adrenergic signaling. Suprisingly, acetoacetate alone lowered cyclic AMP content and isoproterenol was without a detectable effect on cyclic AMP. Nevertheless, in combination the two treatments increased myocardial cyclic AMP content by approximately 30%. Thus acetoacetate bolsters GSH redox potential and may thereby restore one or more of the β-adrenergic signaling proteins proximal to cyclic AMP, *e.g.* β-adrenoceptors, $G_{s\alpha}$, or adenylate cyclase.

The moderate increase in cyclic AMP produced by acetoacetate + isoproterenol was probably insufficient to produce the powerful inotropic actions of the combined treatments. Indeed, acetoacetate's antioxidant mechanisms could restore additional components distal to cyclic AMP. Two potential downstream targets, cyclic AMP–dependent protein kinase A (27) and sarcoplasmic reticular Ca^{2+} ATPase (9, 18) are both inactivated by oxidants. Improved GSH redox potential could reactivate these effector proteins and thereby amplify contractile responses to modest increases in cyclic AMP.

Citrate, glucose 6-phosphate, and antioxidant redox potential. Acetoacetate may enhance GSH/GSSG by increasing myocardial citrate, which promotes metabolic flux through two NADPH-generating pathways. First, the conversion of citrate to isocitrate supplies substrate to the $NADP^+$-dependent isocitrate dehydrogenase reaction (3). Second, citrate inhibits phosphofructokinase (17), causing glucose 6-phosphate to accumulate and diverting glycolytic flux into the NADPH–generating hexose monophosphate shunt. Acetoacetate alone moderately increased myocardial citrate and glucose 6-phosphate contents and $NADPH/NADP^+$ redox state. The combination of isoproterenol and acetoacetate increased these variables more substantially, possibly due to isoproterenol–activated glycogenolysis and glucose uptake (19, 20) which, when combined with citrate inhibition of phosphofructokinase (17), could powerfully increase NADPH formation in the hexose monophosphate shunt.

Impact of phosphorylation potential vs. *glutathione redox state on cardiac function and β-adrenergic inotropism.* Acetoacetate's enhancement of post-ischemic function and its potentiation of β-adrenergic inotropism are strikingly similar to the actions of pyruvate in this model of myocardial stunning (39). Pyruvate increases phosphocreatine phosphorylation potential in parallel with contractile performance leading to the proposal that this energetic enhancement could augment cardiac function by increasing substrate supply to the ATPases that orchestrate the cardiac cycle (7, 8, 10, 28, 29, 43). However, the present findings suggest modification of this working hypothesis, since pyruvate's antioxidant actions could be central to its cardiotonic effect. Acetoacetate did not increase phosphorylation potential of stunned myocardium, yet it increased pre-isoproterenol power just as much as pyruvate. Acetoacetate and pyruvate both potentiated the inotropic effect of isoproterenol to a similar degree (150 ± 10 *vs.* 108 ± 22 $mJ \cdot g^{-1} \cdot min^{-1}$ (39)). Both pyruvate and acetoacetate also increase GSH redox potential. Moreover, *N*-acetylcysteine, a pharmacological antioxidant that doesn't provide fuel for oxidative metabolism, also potentiated isoproterenol's inotropic effects despite a decline in phosphorylation potential (38). The enhancement of the GSH antioxidant system appears to be the principal mechanism of β-adrenergic potentiation by metabolic fuels.

Acetoacetate appears to maintain GSH redox potential by additional, citrate independent mechanisms. Acetoacetate and pyruvate were equally effective at restoring GSH/GSSG although citrate content ($\mu mol \cdot g\ dry^{-1}$) in myocardium treated with pyruvate (8.6 ± 0.8) or pyruvate + isoproterenol (4.8 ± 0.4) (38) exceeded citrate contents in the respective

acetoacetate groups (Figure 6: 1.0 ± 0.2 and 1.9 ± 0.3). Peroxynitrite and hydrogen peroxide are generated in post-ischemic myocardium and rank among the most important prooxidant mediators of cardiac stunning (5, 12). Both compounds convert GSH to GSSG and lower GSH/GSSG redox state: $ONOO^-$ directly oxidizes GSH (12; 23), and GSH is consumed by glutathione peroxidase to detoxify H_2O_2 (13, 26). Alternatively, acetoacetate can consume peroxynitrite in a direct aliphatic nitration reaction yielding a nonreactive derivative, 2-nitroacetoacetate (40). Acetoacetate conversion to acetoacetyl-CoA by 3-oxoacid-CoA transferase (31) may also lessen oxidative stress. Acetoacetyl-CoA detoxifies H_2O_2 generating an unknown reactive oxygen intermediate which is subsequently neutralized by electrons donated by NADH (22). Both of these mechanisms would lower the prooxidant burden on the GSH system, facilitating recovery of GSH/GSSG redox potential.

Limitations. Although the decline in GSH/GSSG ratio indicated prooxidant stress in post-ischemic myocardium, neither the specific prooxidant species nor the biomolecular targets of these compounds were identified or quantified in this investigation. Metabolites were measured in snap-frozen myocardium and expressed as total tissue content, without assessing intracellular metabolite compartmentation. This limitation is most problematic for metabolites like GSH that are sequestered in separate, largely independent mitochondrial and cytosolic pools (21).

The results of this investigation should be extrapolated with caution to the *in vivo* situation. The hearts in this study were isolated and perfused with cell-free crystalloid media. Additional studies are required to determine the impacts of acetoacetate or isoproterenol within the more complex *in vivo* environment. Acetoacetate was tested at 5 mM, a concentration over 100 times its physiological plasma concentration in fasting human subjects (42). The higher concentration was applied to allow direct comparison with pyruvate, which is maximally effective at improving post-ischemic cardiac function at 5 mM (7). The optimum concentration of acetoacetate, and its effectiveness at lower, more physiological concentrations, remain to be determined.

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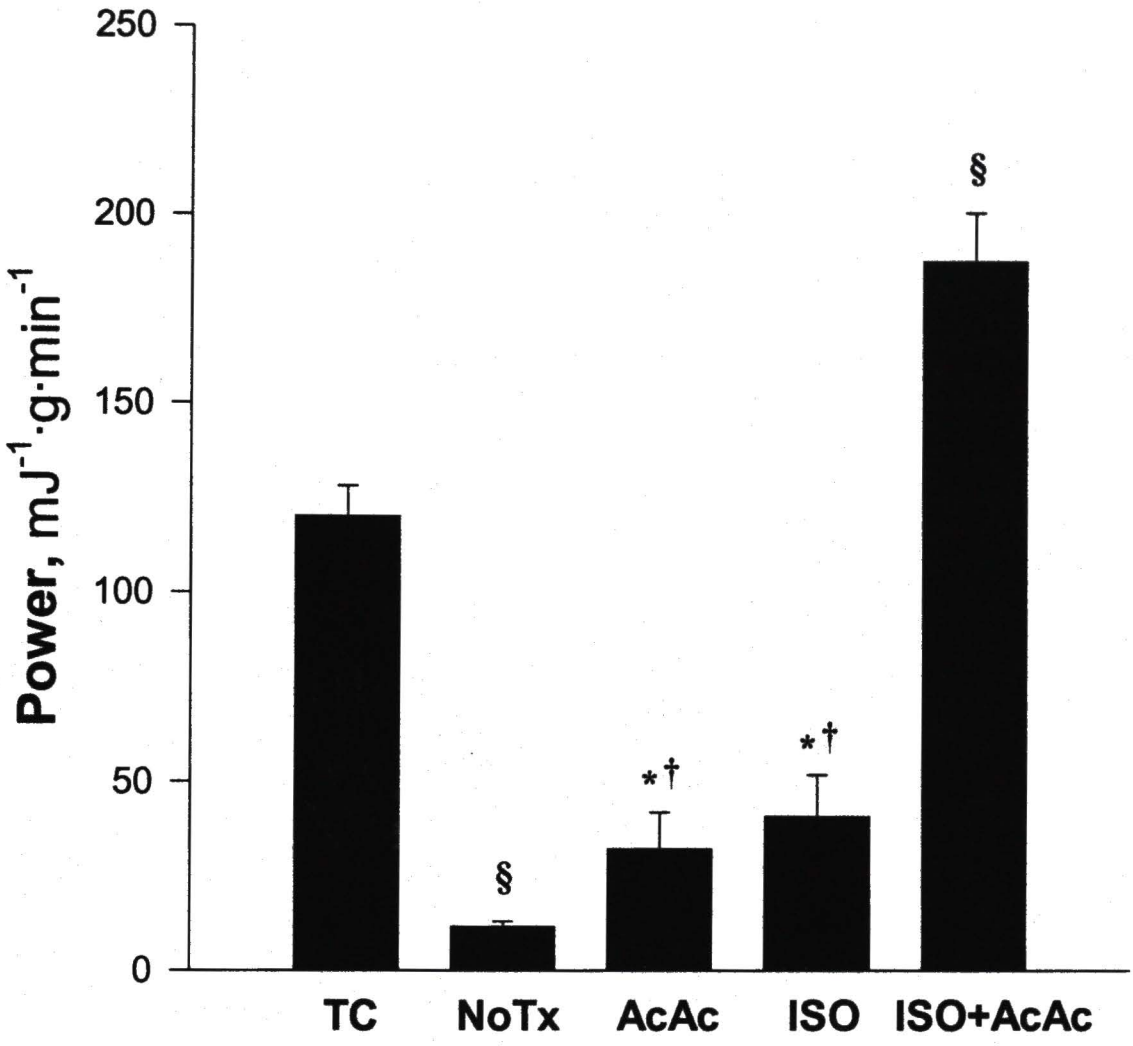

Figure 1. *Cardiac power: impact of stunning and treatment.* Values here and in the other figures are means ± SEM. Power was determined at 45 min reperfusion. Hearts received acetoacetate (AcAc) at 15-45 min, and isoproterenol (ISO) at 30-45 min reperfusion. Values in untreated stunned (NoTx) and nonischemic time control (TC) hearts were obtained at the same perfusion times as the treatment values. *$P<0.05$ *vs.* TC; †$P<0.05$ *vs.* NoTX; §$P<0.05$ *vs.* all groups.

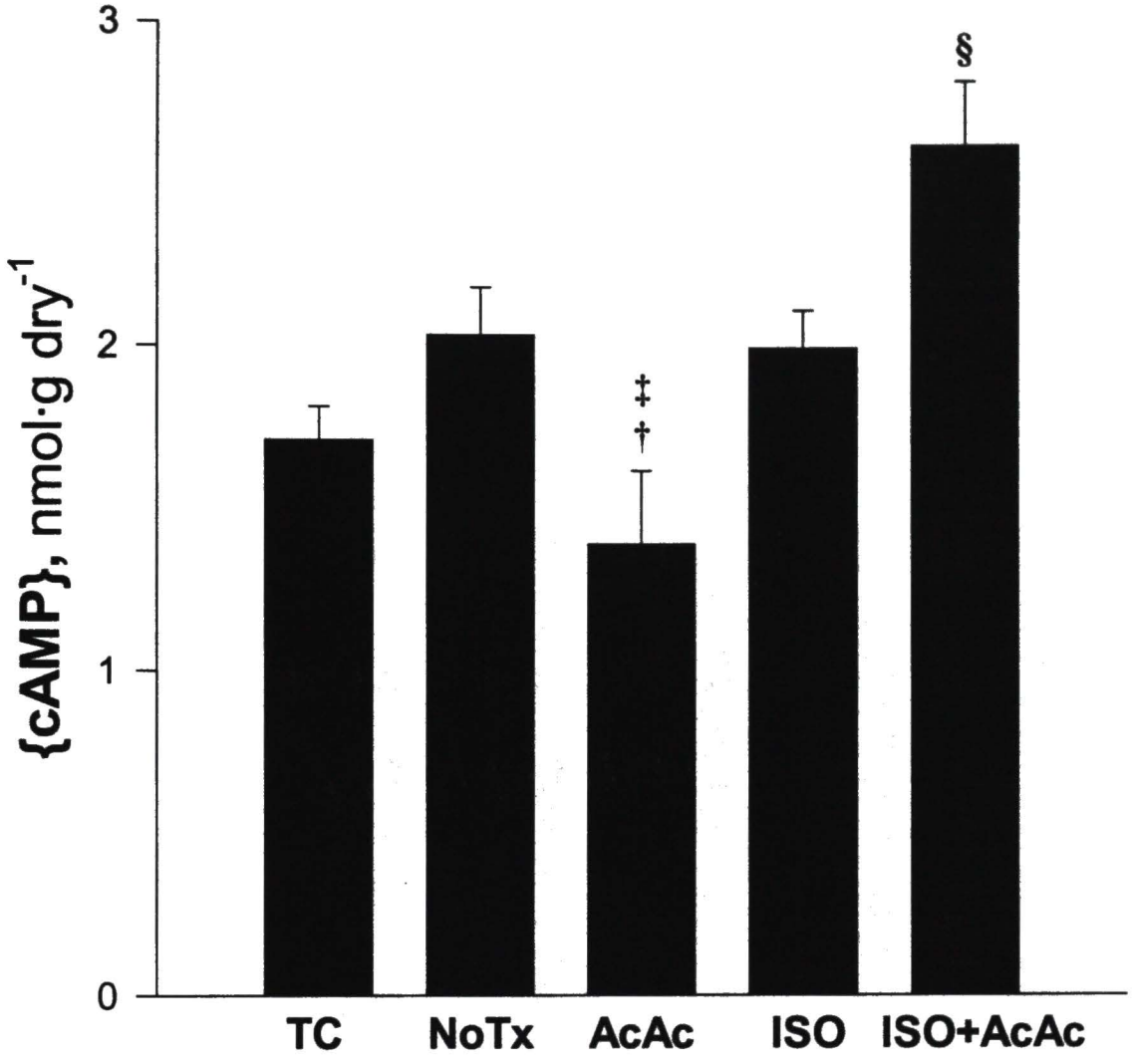

Figure 2. *Myocardial cyclic AMP content.* Cyclic AMP (cAMP) was measured in hearts snap-frozen at 45 min post-ischemia or at 105 min of time control (TC) perfusion. Abbreviations as in Figure 1. $^{\dagger}P<0.05$ *vs.* NoTx; $^{\ddagger}P<0.05$ *vs.* ISO; $^{\S}P<0.05$ *vs.* all groups.

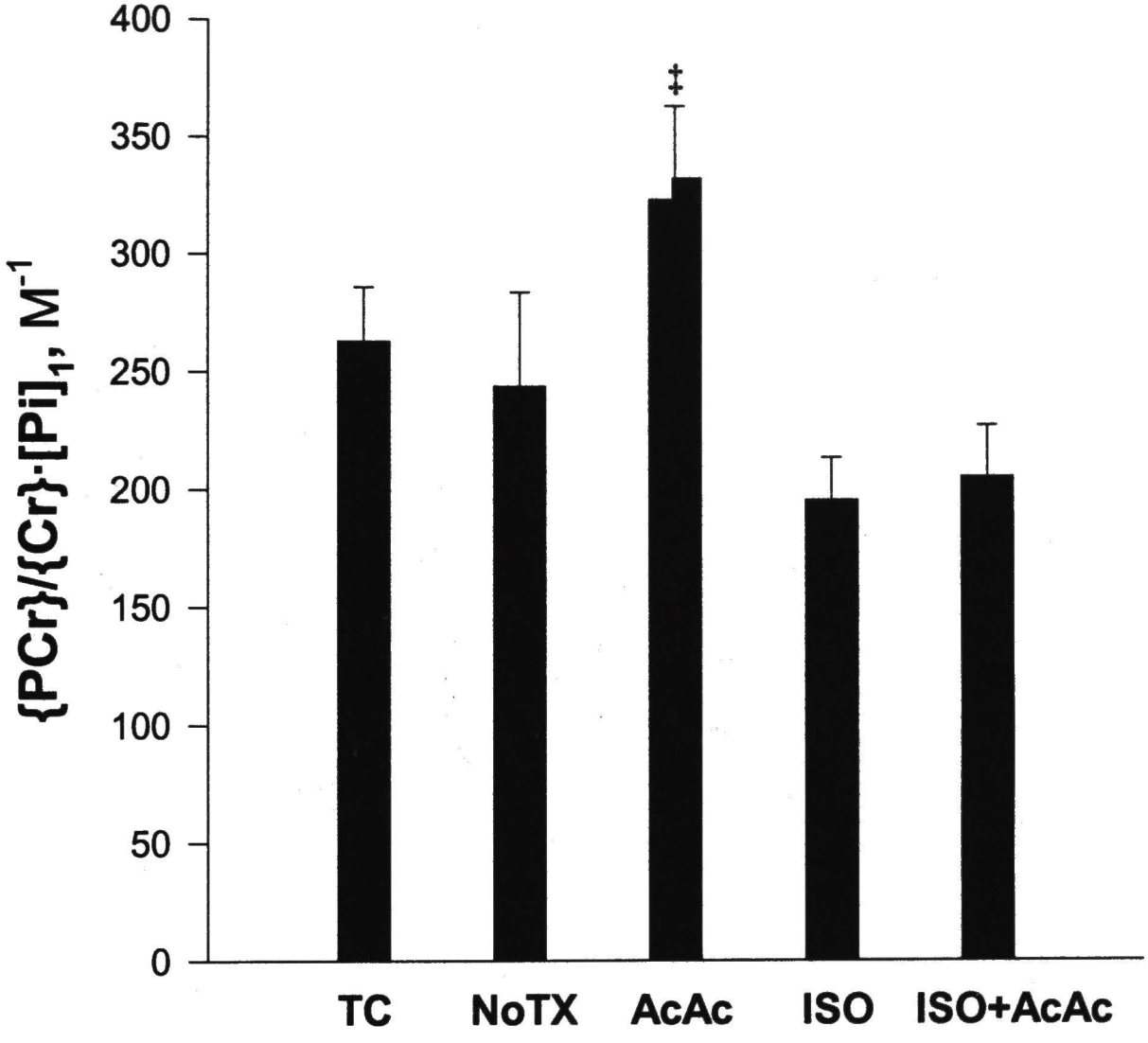

Figure 3. *Myocardial phosphorylation potential.* Myocardial phosphocreatine (PCr) and creatine (Cr) content and inorganic phosphate concentration ($[Pi]_i$) were determined in the same experiments as in Figures 1 and 2. $^{‡}P<0.05$ *vs.* ISO.

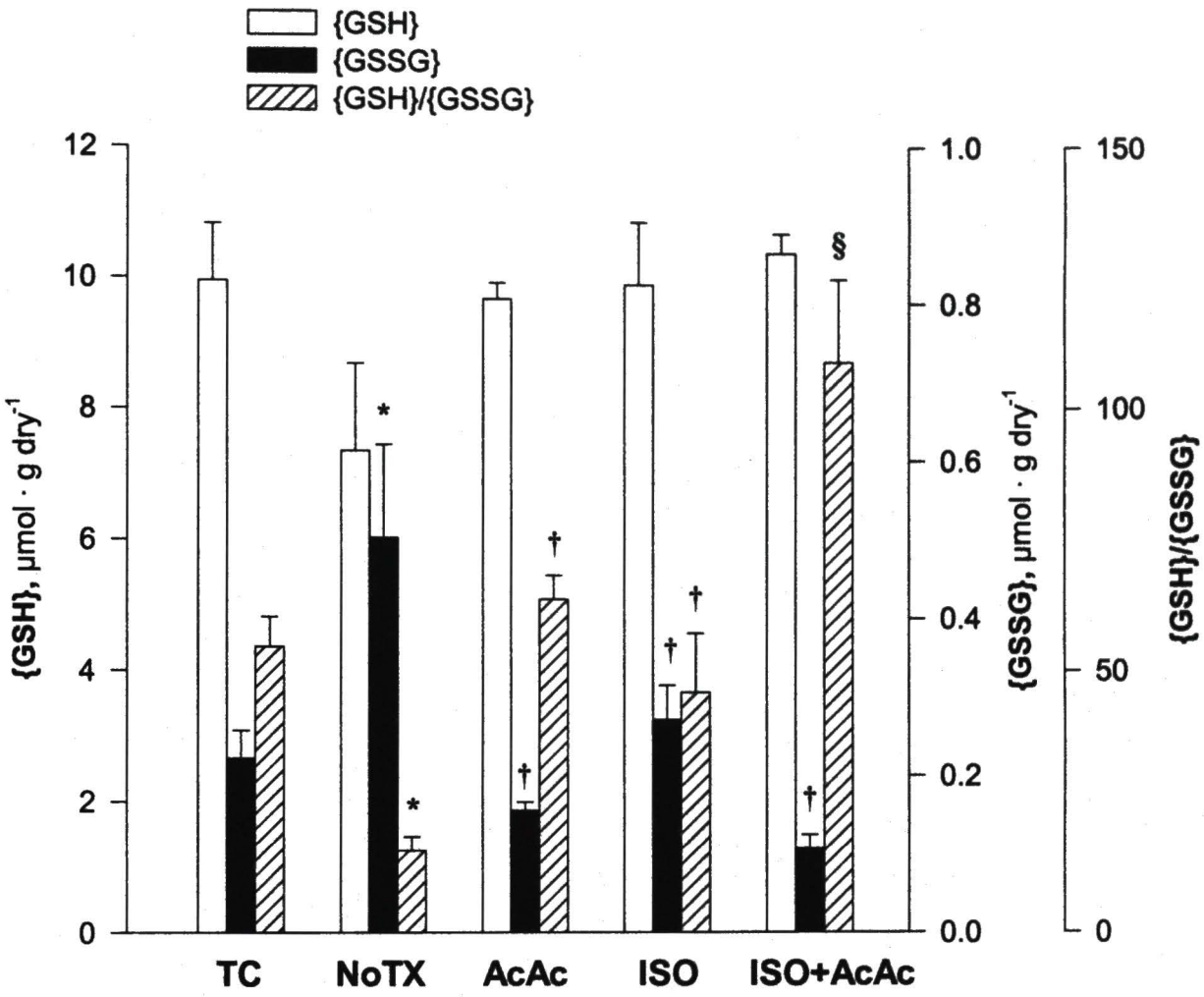

Figure 4. *Glutathione content and redox state.* Myocardial GSH and GSSG contents were measured in the same experiments as in Figures 1-3. Abbreviations as in Figure 1. *$P<0.05$ *vs.* TC; †$P<0.05$ *vs.* NoTx; §$P<0.05$ *vs.* all groups.

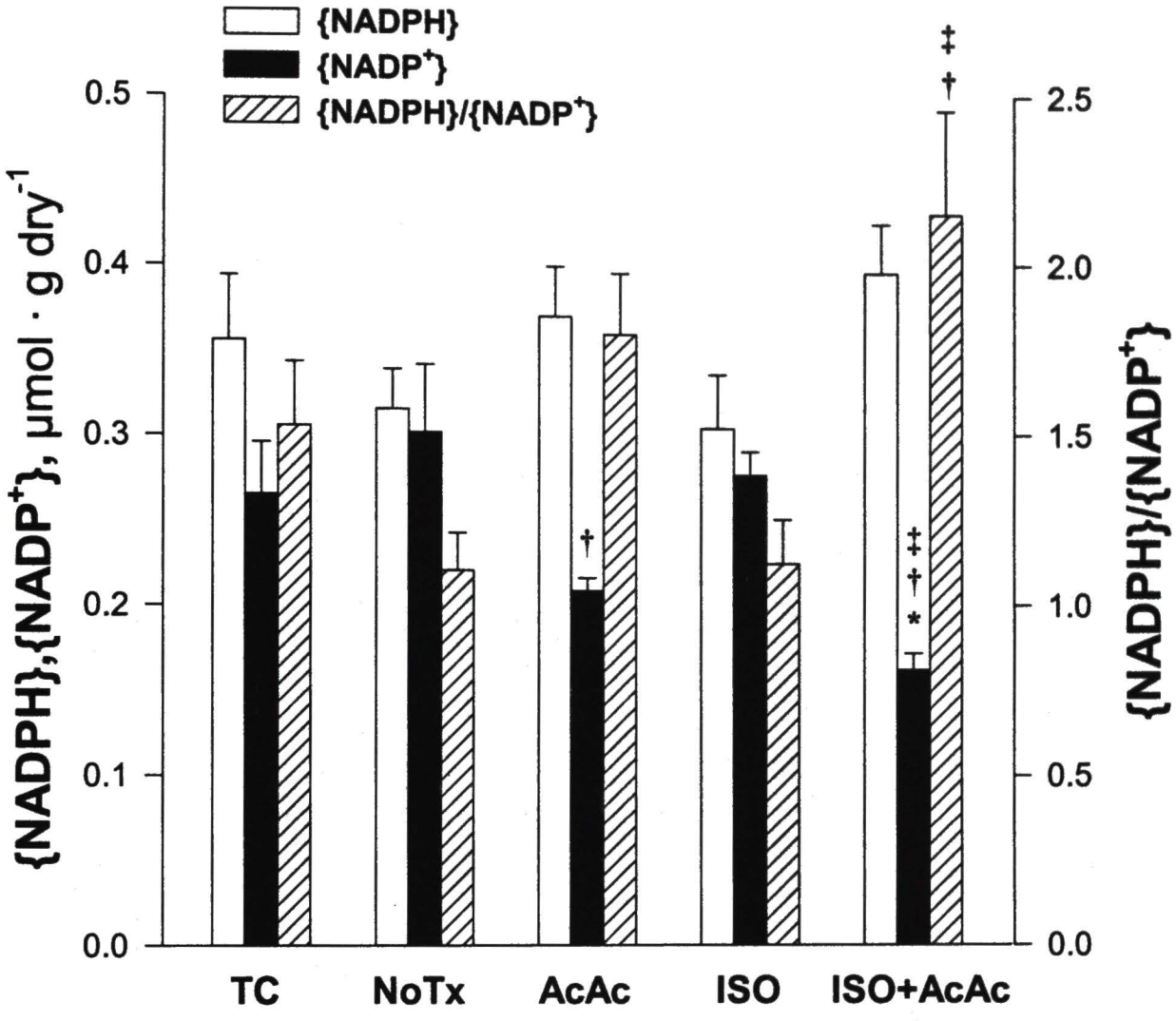

Figure 5. *Myocardial NADPH and NADP*$^+$. NADPH and NADP$^+$ were measured in the same hearts as in Figures 1- 4. *$P<0.05$ *vs.* TC; $^\dagger P<0.05$ *vs.* NoTx; $^\ddagger P<0.05$ *vs.* ISO.

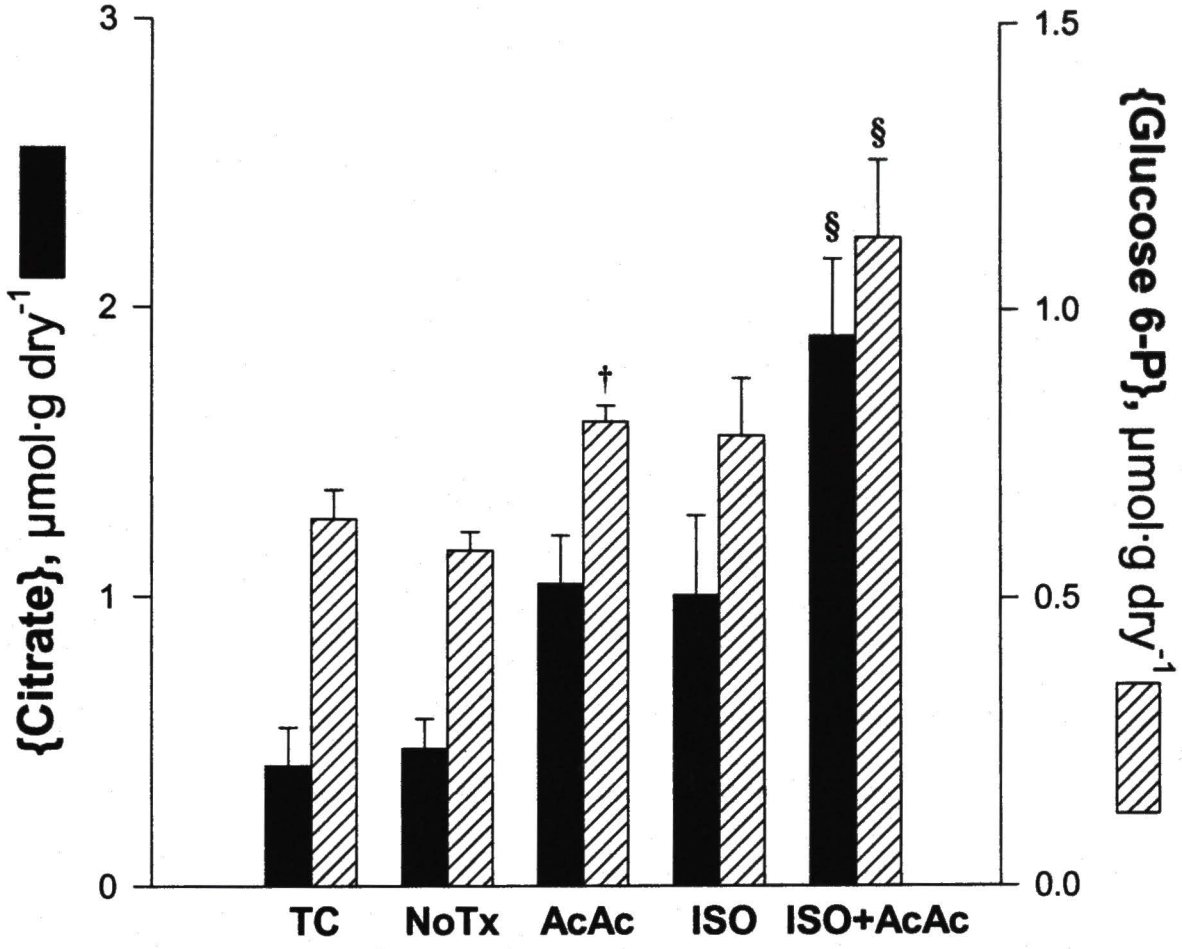

Figure 6. *Citrate and glucose 6-phosphate contents.* Metabolites were measured in the same experiments as in Figures 1-5. $^{\dagger}P<0.05$ *vs.* NoTx; $^{\S}P<0.05$ *vs.* all groups.

CHAPTER IV

CONCLUSION

The first phase of this investigation tested the ability of acetoacetate and β–hydroxybutyrate to restore contractile function and antioxidant redox potential in hearts injured with H_2O_2. Acetoacetate elevated citrate and glucose 6-phosphate content, increased the glutathione antioxidant potential by 160% and almost completely restored cardiac power in H_2O_2-injured myocardium, without altering phosphocreatine phosphorylation potential. β–hydroxybutyrate elevated citrate and glucose 6-phosphate to the same extent as acetoacetate, but increased GSH/GSSG only 60% and failed to improve power. Acetoacetate, but not β-hydroxybutyrate, appeared to have augmented cellular antioxidant redox potential by additional mechanisms independent of citrate and the hexose monophosphate pathway.

At the second phase of this work, acetoacetate was applied to stunned myocardium to determine if its antioxidant actions could revive hearts injured by ischemia/reperfusion and potentiate β–adrenergic inotropism. When given alone, acetoacetate elevated glucose 6-phosphate content, moderately increased power, and restored GSH/GSSG, although it again failed to increase phosphorylation potential. Isoproterenol

generated a similar increase in power, and augmented GSH/GSSG more modestly. When used in combination, isoproterenol and acetoacetate synergistically enhanced cardiac power and cyclic AMP content in parallel with glutathione redox state. This combination of inotropic and metabolic treatments bolstered NADPH/$NADP^+$ redox state, the source of reducing power to maintain GSH/GSSG, and increased cyclic AMP, citrate, and glucose 6-phosphate contents. Thus, acetoacetate in combination with isoproterenol, activated metabolic mechanisms to increase NADPH and, thus, GSH redox potentials.

These novel findings support the following conclusions.

1. Acetoacetate enhanced contractile performance of metabolically challenged myocardium by augmenting the glutathione redox state.
2. Despite its similar chemical structure and metabolic fate, β–hydroxybutyrate was a less effective antioxidant than acetoacetate, and, consequently, failed to improve cardiac performance.
3. Acetoacetate enhanced myocardial sensitivity to β–adrenergic stimulation, possibly by enhancing glutathione antioxidant redox potential.

The antioxidant properties of acetoacetate and β–hydroxybutyrate in myocardium have never been reported. Furthermore, this is the first evidence demonstrating acetoacetate's favorable effects on cardiac stunning and prooxidant injury.

IMPLICATIONS AND INDICATIONS FOR FUTURE RESEARCH

Acetoacetate's antioxidant actions and its interaction with β–adrenergic agents in stunned hearts suggest that it may be beneficial as a treatment for cardiac insufficiency in patients. Acetoacetate's enhancement of myocardial β–adrenergic sensitivity may lower the dosages of catecholamines required to improve cardiac performance, and thereby mitigate the risk of arrhythmias associated with the use of these agents (Mertes, 1993). Acetoacetate may also ameliorate the oxidative stress characteristic of certain disease states. Diabetes is particularly devastating because it inflicts severe, widespread oxidative damage to numerous tissues. Plasma acetoacetate concentrations are sharply increased in poorly managed diabetics. The results of this investigation suggest that acetoacetate could conceivably alleviate the prooxidant burden associated with untreated diabetes and prolong the degenerative progression of the disease. Whether acetoacetate's antioxidative action requires glycolysis, which is inhibited in diabetes, is unknown.

Acetoacetate's specific antioxidative mechanisms have yet to be confirmed. The contribution of acetoacetate's direct neutralization of peroxynitrite and H_2O_2, versus its capacity to elevate $NADPH/NADP^+$ and enhance the glutathione redox state, remains undetermined. In this regard, direct measurements of acetoacetyl-CoA, which neutralizes H_2O_2, would be valuable. Acetoacetyl CoA content in myocardium is below the detection limit of colorimetric assays, so more sensitive techniques are required to measure this metabolite. Pharmacological inhibition of the processes generating reducing

equivalents for glutathione reductase may help delineate the impact of acetoacetate's enhancement of the NADPH/GSH antioxidant mechanism.

A limitation in this study was the inability to determine the specific cellular components damaged by H_2O_2 and stunning. Establishing which proteins and phospholipids are protected would be valuable to delineate acetoacetate's mechanisms of β–adrenergic potentiation and cardioprotection. Measurement of peroxynitrite or its oxidation products could help determine if this prooxidant is generated in the ischemically challenged myocardium, and if acetoacetate alleviates oxidative stress by neutralizing this compound. Furthermore, to begin assessment of acetoacetate's potential as a cardioprotectant in the diabetic setting, and to determine whether glucose is a mandatory co-substrate for acetoacetate's salutary actions, experiments could be conducted utilizing acetoacetate alone or as a co-substrate with fatty acids.

APPENDIX

Cyclic AMP radioimmunoassay for dog and guinea pig myocardial tissue

Procedure

Day 1 Extraction

Vials containing pulverized heart tissue must be kept frozen in liquid nitrogen. Approximately 100 mg of powdered tissue is transferred into pre-weighed homogenization tubes containing 2 ml of 6% Trichloroacetic acid (TCA), and weighed. The mass of the empty homogenization tube, the tube + TCA, and the tube + TCA + tissue must be determined. The tissue/TCA mix is homogenized for 1 min with a cooled, rotating teflon piston. The homogenate is centrifuged for 10' at 1200 g (3,200 rpm). The supernatant is decanted into 10 ml graduated screw-cap tubes on ice. The pellet is discarded. The supernatant is extracted three times with 5 ml of H_20 saturated ether. The tubes are then kept overnight uncapped and on ice to allow residual ether to evaporate from the samples.

Day 2 Freeze-dry

Extract volume is brought to 5 ml by adding water to the graduated screw-top tubes. The tubes are vortexed and kept on ice. Aliquots (1 ml) of extract are transferred into labeled freeze-dry tubes. The tubes are then frozen for approximately 20 min at –90°C. The tubes are quickly transferred from the freezer to a centrifugal vacuum evaporator (savant) cooled

below -90°C prior to centrifuge loading. The centrifugation is begun and when maximal speed is achieved, the vacuum is applied. The samples are left to dry overnight.

Day 3 1° Antibody

Standard cAMP solutions are generated accordingly.

#1		1 mg/ml stock in PBS-gel
#2 High STD	10 pmol/µl	36.92 µl of #1 + 9.963 ml PBS-gel
#3 Med STD	0.1 pmol/µl	100 µl of #2 + 9.900 ml PBS-gel
#4 Low STD	0.01 pmol/µl	1000 µl of #3 + 9.000 ml PBS-gel

The three standards above are used to generate the standard binding curve when mixed with antibody and radioiodinated cyclic AMP. To correct for non specific I125-cAMP binding, normal rabbit serum (NRS) is substituted for the cAMP antibody in triplicate (tube #2). Standards are generated in triplicate to adjust for variability.

	Tube#	PBS-gel (µl)	STD/Sample (µl)	1'Antibody (µl)	I125 (µl)
STD	1	0	0	0	100
STD	2	500	0	*200µl NRS	100
STD	3	500	0	200	100
STD	4	497.5	(Low #4) 2.5	200	100
STD	5	490	(Low #4) 10	200	100
STD	6	475	(Low #4) 25	200	100
STD	7	400	(Low #4) 100	200	100
STD	8	250	(Low #4) 250	200	100
STD	9	400	(Med #3) 100	200	100
STD	10	250	(Med #3) 250	200	100

STD	11	490	(High #2) 10	200	100
STD	12	475	(High #2) 25	200	100
Control	13	450	50	200	100
Sample	14→	500	0	200	100

Tubes are vortexed and incubated at room temperature under parafilm for 12 or more hours.

Day 4 2° Antibody

All tubes receive 200 μl of 2° antibody except the total tubes (sample tubes #1). The tubes are refrigerated (4°C) overnight.

Day 5 Pour off

Sample tubes #1 do not receive polyethylene glycol (PEG), are not centrifuged and are not "poured off". Tubes are loaded in centrifuge racks. PEG (2 ml) is added to the tubes and the racks are immediately refrigerated for 20 min just prior to centrifugation. The tubes spin at 3,200 rpm for 15 min. The radioactive supernatant is carefully decanted and discarded. The tubes are then capped and the bound radioactivity is determined by gamma spectrometry. A standard curve is generated by graphing % binding against the log of the concentration of the standards (#3-#12). To determine percent binding, the difference between the average counts for a given sample set and the non-specific binding (#2) is first determined. This value is then divided by the maximal binding (#3 minus #2) to ascertain %binding. The percentage binding of each unknown sample is extrapolated against the standard curve to determine the average picomoles in a given

tube. These values are then converted for the volume assayed to determine the amount of cyclic AMP in the myocardial extract. The amount of cyclic AMP per gram of dry tissue is then determined by considering the amount of wet tissue used to generate extract and the tissue's wet/dry ratio.

Table 1. *Impact of H_2O_2 and ketone bodies on hemodynamic variables*

	Pre-H_2O_2		10' H_2O_2		30' post-H_2O_2		60' treatment	
	HR	CO	HR	CO	HR	CO	HR	CO
NoTx	230±6.9	22.3±1.7	220±16.0	14.9±1.5	205±8.8	8.0±1.4	196±11	7.1±1.3
AcAc	201±7.5	17.8±1.3	206±5.1	10.3±0.6	205±7.4	8.2±0.8	215±5.4	11.9±1.6
β-OHB	208±3.1	18.7±2.0	202±8.2	11.8±0.7	199±10.8	7.8±0.9	193±0.5	7.3±0.7

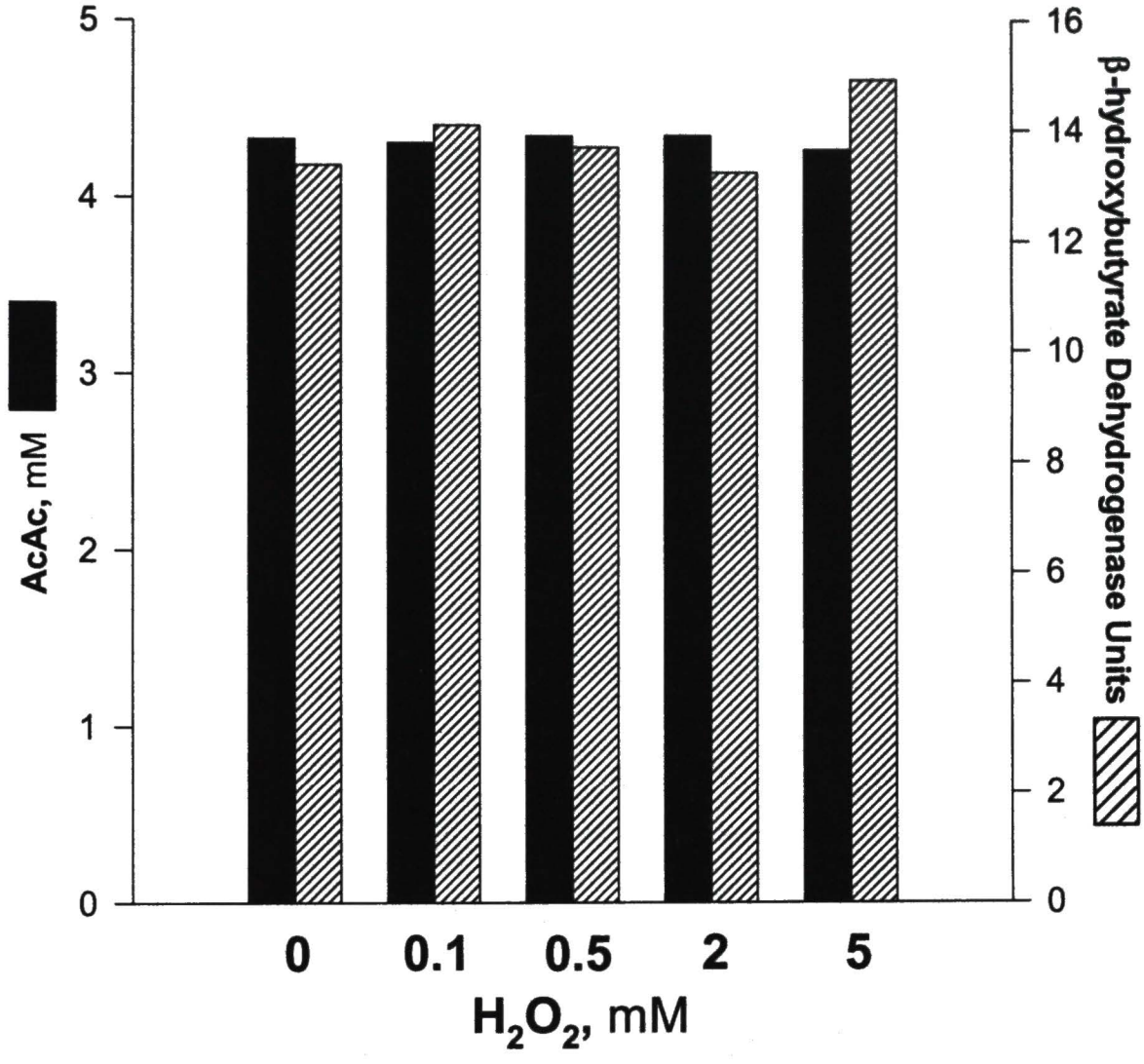

Figure 1. *Acetoacetate-H_2O_2 neutralization assay.* Acetoacetate was measured following introduction of a series of H_2O_2 concentrations. β-hydroxybutyrate dehydrogenase activity is expressed as the rate of NADH formation.

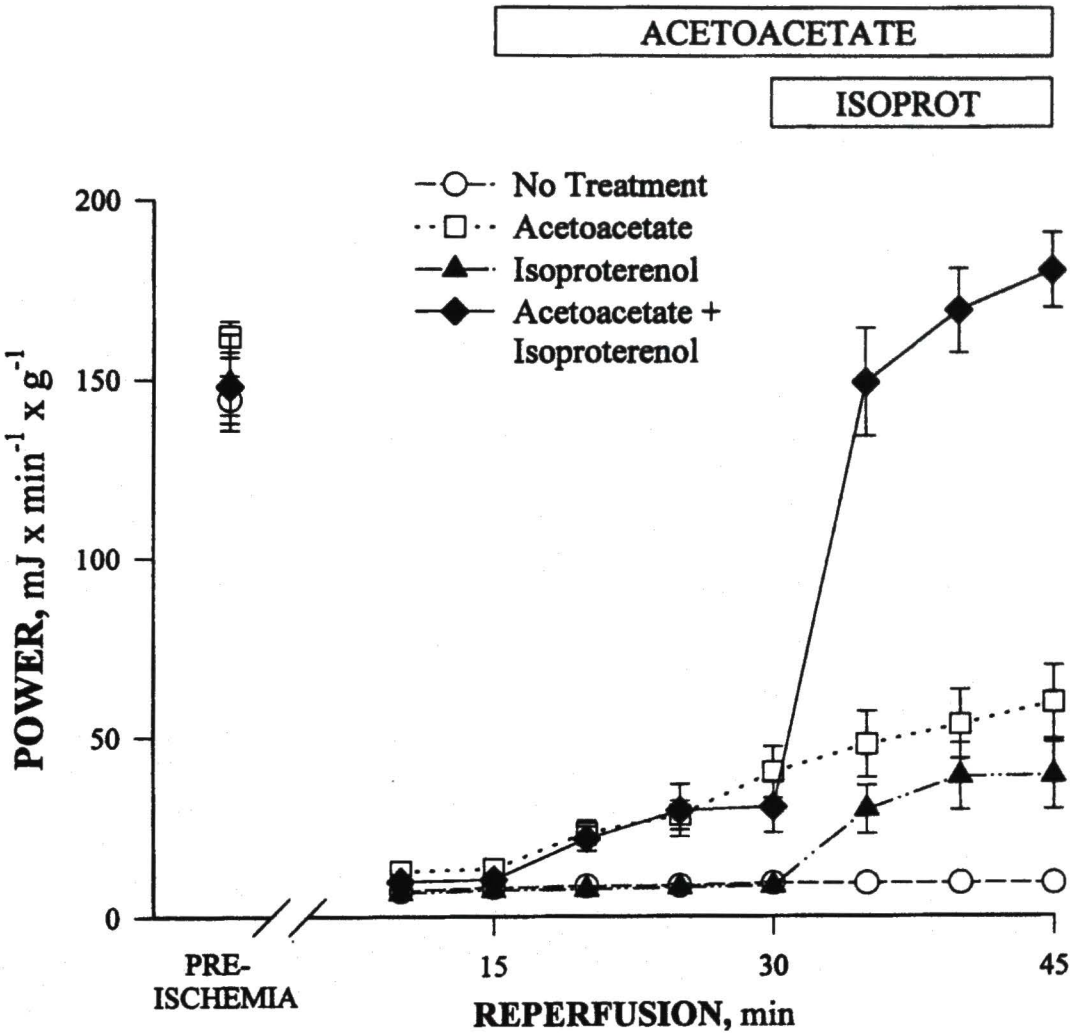

Figure 2. *Cardiac power: impact of treatment during reperfusion.* Cardiac power was measured at pre-ischemia baseline and at 5 min intervals during reperfusion following 45 min low-flow ischemia. Acetoacetate and isoproterenol were administered during the periods indicated by the bars at the top of the figure.

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