Acetaminophen in the Hypoxic and Reoxygenated Guinea Pig Myocardium

TYLER H. RORK,* KNOX VAN DYKE,† NORELL M. SPILER,* AND GARY F. MERRILL*,[1]

*Division of Life Sciences, Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, New Jersey 08854; and †Department of Biochemistry and Molecular Pharmacology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, West Virginia 26506

We investigated the effects of 0.35-m*M* acetaminophen and its vehicle on isolated, perfused guinea pig hearts made hypoxic and subsequently reoxygenated. Hearts were allowed 30 min postinstrumentation to reach baseline, steady-state values, and then were exposed to 6 min of hypoxia (5% O_2, 5% CO_2, balance N_2) followed by 36 min of reoxygenation (95% O_2, 5% CO_2). We recorded hemodynamic, metabolic, and mechanical data in addition to assessing ultrastructure and the capacity of coronary venous effluent to reduce reactive oxygen species. We found that acetaminophen-treated hearts retained a greater fraction of mechanical function during hypoxia and reoxygenation. For example, the average percentage change from baseline of left ventricular developed pressure in acetaminophen- and vehicle-treated hearts at 6 min reoxygenation was 9 ± 2% and −8 ± 5% ($P < 0.05$), respectively. In addition, electron micrographs revealed greater preservation of myofibrillar ultrastructure in acetaminophen-treated hearts. Biochemical analyses revealed the potential of coronary effluent from acetaminophen-treated hearts to significantly neutralize peroxynitrite-dependent chemiluminescence in all recorded time periods. During early reoxygenation, the percentage inhibition of peroxynitrite-mediated chemiluminescence was 56 ± 10% in vehicle-treated hearts and 99 ± 1% in acetaminophen-treated hearts ($P < 0.05$). We conclude that acetaminophen has previously unreported cardioprotective properties in the nonischemic, hypoxic, and reoxygenated myocardium mediated through the reduction of reactive oxygen species. Exp Biol Med 229:1154–1161, 2004

Key words: Langendorff; ventricular function; peroxynitrite; chemiluminescence; cardioprotection

Introduction

The effects of acetaminophen in the mammalian cardiovascular system have not been rigorously examined (1). In recent investigations, we have shown that acetaminophen exhibits cardioprotective efficacy in the mammalian myocardium under conditions of ischemia and reperfusion (2–6). The cardioprotective properties of acetaminophen are mediated by the antioxidant nature of the drug [i.e., it has the ability to reduce reactive oxygen species such as hydroxyl radical and peroxynitrite (4)].

Although acetaminophen exhibits cardioprotective effects in ischemia and reperfusion, it is not known whether this effect translates to a more specific hypoxia and reoxygenation environment. Karmazyn *et al.* (7) reported beneficial effects of nonsteroidal anti-inflammatory drugs on the hypoxic mammalian myocardium, but did not test acetaminophen (not an nonsteroidal anti-inflammatory drug). Teng *et al.* (8) found that phenols such as urate protect the hypoxic myocardium by reducing the damaging influence of peroxynitrite. The ability of acetaminophen (a phenol) to preserve mechanical function and attenuate myocardial damage in hypoxia and reoxygenation may therefore be similar to the aforementioned studies.

The purpose of this investigation was to examine the actions of acetaminophen in the nonischemic hypoxic and reoxygenated mammalian myocardium. We investigated hemodynamic, metabolic, and mechanical variables, as well as assessed myofibrillar ultrastructure and reactive oxygen species-mediated chemiluminescence. It is well known that hypoxia and reoxygenation cause an overload of reactive oxygen species in heart tissue (9). We have previously shown that acetaminophen acts to reduce these damaging species in ischemia and reperfusion, and we therefore hypothesized that acetaminophen would be similarly cardioprotective in a hypoxia and reoxygenation environment.

Materials and Methods

Animals and Langendorff Preparation. After IACUC and IRB review and approval, Hartley strain male guinea pigs (375 ± 25 g) were obtained from Charles River Laboratories (Wilmington, MA). They were allowed to

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[1] To whom correspondence should be addressed at Division of Life Sciences, Department of Cell Biology and Neuroscience, Rutgers University, 604 Allison Rd., Piscataway, NJ 08854. E-mail: merrill@biology.rutgers.edu

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acclimate several days before being brought to the laboratory. Guinea pigs were killed, a bilateral thoracotomy was performed, and hearts were instrumented and perfused *in situ* as described by Bunger *et al.* (10, 11). Hearts were subsequently removed from the mediastinum and attached to a perfusion apparatus; elapsed time from euthanasia to initiation of perfusion *in vitro* was 3–4 min. Instrumentation included excision of the left atrial appendage and advancement of a flaccid latex balloon across the mitral valve and into the left ventricle to monitor mechanical variables; the balloon was filled with Krebs-Henseleit buffer (KHB) to an end-diastolic pressure of 0–5 mmHg (volume of 75–100 μl). We also placed a large-bore catheter in the trunk of the pulmonary artery to collect coronary venous effluent for analysis of metabolic data and creatine kinase activity. Pacing electrodes were placed at the base of the right ventricle, and the hearts were paced at 240 beats per minute (model S44 stimulator, Grass-Telefactor, West Warwick, RI). Physiologic heart temperature was confirmed by passing a 0.025 inch-diameter thermistor probe into the right ventricle (model BAT-12, Physitemp, Clifton, NJ). Hearts were perfused retrogradely via the cannulated aorta (i.e., antegrade coronary perfusion).

On completion of instrumentation, hearts were perfused at a constant pressure of approximately 50 mmHg following procedures previously described in this laboratory (12–14). Coronary perfusate flow was allowed to vary naturally. Hearts were allowed 30 min postinstrumentation for monitored variables to achieve steady-state conditions. Monitored variables included heart rate (beats per minute), coronary perfusate flow (CPF; ml/min per gram), coronary perfusion pressure (CPP; mmHg), left ventricular developed pressure (LVDP; mmHg), its first derivative ($\pm dP/dt_{max}$; mmHg/s), and perfusate gases and pH. Myocardial oxygen consumption (MVo_2; μl/min per gram) was calculated as the product of arterial–venous O_2 content and CPF (15). Coronary vascular resistance (CVR) was calculated as the quotient: CPP (mmHg)/CPF (ml/min/g) = CVR (mmHg/ml/min/g), the pressure rate product was calculated as heart rate (beats per minute) × LVDP (mmHg) = pressure rate product (mmHg/min), and myocardial efficiency was calculated as the quotient: $+dP/dt_{max}$ (mmHg/s)/MVo_2 (μl/s/g) = myocardial efficiency (mmHg/μl/g).

Perfusate and Perfusion. Perfusate was a modified KHB physiologic salt solution warmed to 38°C and containing (in millimoles) 128.0 NaCl, 4.7 KCl, 1.5 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 24.9 $NaHCO_3$, 10.0 glucose, 2.0 pyruvate, and 200 μU/ml insulin. Acetaminophen (0.35 m*M*) or its vehicle (KHB) was added directly to the perfusate reservoir at the commencement of the experiment, as previously reported (5). This concentration corresponds to approximately 50 μg/ml in human circulating plasma (6), or two to five times the therapeutic dose for analgesia or antipyresis (1). This dose is well below the approximate 300 μg/ml concentration considered potentially cytotoxic (1). Retrograde aortic flow (antegrade coronary flow) was established incrementally by controlling CPP hydrostatically. Flow was delivered from one of two 500-ml water-jacketed reservoirs and was continuously monitored ultrasonically (model T101 flowmeter, Transonic Systems, Ithaca, NY). As needed, one reservoir was equilibrated with a gas mixture containing 95% O_2 and 5% CO_2 (normoxia), and the other was filled with an experimental mixture of gases (see following).

Left ventricular pressures were measured isovolumetrically, and perfusate samples were obtained anaerobically using 1.0-ml tuberculin syringes. Standard electrodes were used to measure pH, Pco_2 (mmHg), Po_2 (mmHg), and base excess (Chiron Diagnostics model 248 blood gases/pH analyzer, Bayer Diagnostics, Norwood, MA). Arterial and venous oxygen contents were calculated as the product of Po_2 and the solubility of oxygen in salt solution at 38°C, as previously reported (12, 13, 16); the solubility coefficient was 2.28×10^{-2} μl/ml/mmHg (15). A data acquisition system (iWorx model 214, CB Sciences, Dover, NH) in series with a personal computer (Compaq Evo running LabScribe software version 6.0) was used to record monitored variables. After hearts reached baseline, steady-state conditions (i.e., after 30 min normoxic perfusion), hearts were exposed to 6 min of hypoxia (5% O_2, 5% CO_2, balance N_2), followed by 36 min of normoxic reoxygenation, as previously described (14).

Experimental Protocols. *Hemodynamic and Mechanical Properties.* The purpose of this protocol was to determine the effects of hypoxia and reoxygenation in the absence ($n = 10$) and presence ($n = 10$) of acetaminophen on the hemodynamic and mechanical status of the isolated guinea pig heart. Data for hemodynamic and mechanical variables were collected at baseline, 6 min of hypoxia, and 6 and 36 min of reoxygenation.

Metabolic Properties and Creatine Kinase Release. Vehicle-treated ($n = 10$) and acetaminophen-treated ($n = 10$) hearts were used to monitor the release of creatine kinase (CK) and other variables indicative of general tissue metabolism. Creatine kinase was measured using standard assays (product CK-NAC, Stanbio Laboratory, Boerne, TX) as previously described by Szasz (17) and Rosalki (18). Briefly, 1 ml of reconstituted reagent was pipetted into a 1-ml minimum cuvet and incubated at 37°C for 5 min. Subsequently, 25 μl of sample was added to the cuvet and again incubated for 2 min. The cuvet was then placed in a spectrophotometer (Jenway 6300, Jenway Limited, Essex, England), and the increase in absorbance was read at 60-sec intervals for a total of 3 min. Creatine kinase activity was determined as

$$\text{CK activity (U/l)} = \frac{\Delta \text{ absorption/min}}{\text{absorptivity}} \times \frac{\text{total volume}}{\text{sample volume}}.$$

Perfusate gases, pH, and other metabolic variables were measured as described above. Samples were collected at

baseline (30 min), 6 min of hypoxia, and 6 and 36 min of reoxygenation.

Myofibrillar Ultrastructure. Myofibrillar ultrastructure was assessed using electron microscopy in acetaminophen-treated ($n = 2$ at each time period) and vehicle-treated ($n = 2$ at each time period) hearts. Hearts were exposed to the same experimental perfusion as stated above; however, they were perfused with Trump's fixative (pH 7.2) for 2 min under steady state conditions (i.e., 30 min of perfusion with normoxic KHB), at 6 min of hypoxia, and at 36 min reoxygenation after 6 min of hypoxia. Hearts were then submerged in Trump's fixative, and 1–2-mm^3 blocks of myocardium were excised from the anterior free wall of the left ventricle midway between the left ventricular and left anterior descending branches of the left main coronary artery, equidistant from base to apex, as previously described (2). Blocks were postfixed with 1% osmium tetroxide and subsequently dehydrated in graded ethanol. Samples were embedded in Epon-Araldite cocktail, sectioned with a diamond knife ultramicrotome (model LKB-2088, LKB, Bromma, Sweden), and viewed with an electron microscope (model JEM-100CXII, JEOL USA, Peabody, MA), using standard methods (19).

Reactive Oxygen Species. The interference of peroxynitrite-mediated luminol oxidation and superoxide-mediated lucigenin oxidation was assessed in coronary venous effluent from vehicle-treated ($n = 7$) and acetaminophen-treated ($n = 7$) hearts. In addition, the samples were assessed for total nitrite content by colorimetric methods. Perfusate samples were stored at –80°C until analysis. For the peroxynitrite-mediated luminol oxidation assay, 100 μl of each sample was treated with 100 μl of luminol (final concentration of 0.6 m*M*), 100 μl of 3-morpholinosydnominine (SIN-1; final concentration 5.8 m*M*), and 200 μl of phosphate buffered saline. The samples were mixed and, pipetted into 3-ml round-bottom luminometer tubes and immediately placed in a temperature-controlled (37°C) luminometer (model LB9505C, Berthold Technologies, Bad Wildbad, Germany). Each sample was analyzed for 20 min, and the light generated was acquired, plotted, and integrated with a personal computer running KINB software. The assay was reported as counts per minute integrated over the 20-min period, as previously reported by Van Dyke *et al.* (20) and Merrill (4). All samples were statistically compared to standard control assays.

The methods for determination of lucigenin dependent chemiluminescence by xanthine/xanthine oxidase are based on published reports of Li *et al.* (21). For chemiluminescent analysis, 100 μl of each sample was treated with 100 μl of xanthine (final concentration of 0.5 m*M*), 100 μl of xanthine oxidase (final concentration of 4 μg/ml), 100 μl of lucigenin (final concentration of 0.6 m*M*), and 100 μl phosphate buffered saline. Analysis, measurement, and statistical comparison of superoxide-mediated lucigenin oxidation were identical to that of peroxynitrite-mediated luminol oxidation.

The colorimetric method for the determination of NO concentration is based on the reports of Schmidt (22) and was performed using a standard assay kit (BIOXYTECH nitric oxide assay, Oxis, Inc., Portland, OR). For colorimetric assays, 5 μl reconstituted nitrate reductase enzyme was mixed with 5 μl coronary effluent sample, 80 μl phosphate buffered saline, and 10 μl NADH (final concentration 2 m*M*). Fifty microliters sulfanilamide (p-aminobenzenesulfonamide) in 3*N* HCl was subsequently added, and the solution was briefly mixed. Fifty microliters N-(1-naphthyl) ethylenediamine dihydrochloride in deionized water (0.1%) was then added, and the solution was mixed for 5 min at room temperature. Solutions were then placed in a microtiter plate reader (SpectraMax 96-well spectrophotometer, Molecular Devices, Sunnyvale, CA), and absorbance was measured at 540 nm. Standard curves were constructed, and acetaminophen-treated samples were compared with matched vehicle-treated samples.

Collection of coronary venous effluent for analysis of peroxynitrite, superoxide, and nitric oxide occurred at baseline, 6 min hypoxia, and 3 and 36 min of reoxygenation.

Statistical Analysis. Student's *t*-test for unpaired data was used to analyze differences in treatment means between treatment groups. Statistically significant differences were established at $P < 0.05$, and all data are reported as mean ± SE.

Results

Hemodynamic and Mechanical Properties. There were no significant differences in CPF, CPP, and CVR between treatment groups under baseline conditions or during hypoxia (Table 1). During reoxygenation, CVR differed modestly, but significantly, between the two treatment groups. For example, the average CVR of vehicle and acetaminophen-treated hearts at 6 min reoxygenation was 8.8 ± 0.7 and 6.5 ± 0.5 mmHg/ml/min/g ($P < 0.05$), respectively (Table 1).

There were significant differences in acetaminophen-treated versus vehicle-treated hearts in both the maintenance and recovery of mechanical function during hypoxia and reoxygenation. For example, LVDP was attenuated to a lesser degree during hypoxia in acetaminophen versus vehicle-treated hearts (–46 ± 4% vs. –62 ± 3%, respectively; $P < 0.05$; all values are expressed as percentage change from baseline). Acetaminophen-treated hearts improved similarly during reoxygenation (Fig. 1). Acetaminophen-treated hearts also showed increased amplitude of $+dP/dt_{max}$ during hypoxia (–33 ± 5% vs. –57 ± 3%; $P < 0.05$), early reoxygenation (3 ± 4% vs, –17 ± 6%; $P < 0.05$), and late reoxygenation (28 ± 6% vs. –5 ± 5%; $P < 0.05$; Fig. 2). The $-dP/dt_{max}$ was also significantly improved in acetaminophen- versus vehicle-treated hearts during reoxygenation (data not shown). In addition,

Table 1. Influence of Hypoxia and Reoxygenation with or without Acetaminophen Treatment on Hemodynamic Variables in the Isolated Guinea Pig Heart as a Function of Time

	Baseline	6 min hypoxia	6 min reoxygenation	36 min reoxygenation
CPP (mmHg)				
Vehicle	54 ± 0.4	53 ± 0.4	54 ± 0.4	53 ± 0.3
Acetaminophen	53 ± 0.3	52 ± 0.3	52 ± 0.4	53 ± 0.3
CPF (ml/min/g)				
Vehicle	7.2 ± 0.4	18.8 ± 1.0	7.0 ± 0.5	7.0 ± 0.1
Acetaminophen	7.6 ± 0.3	17.8 ± 1.1	8.6 ± 0.6	7.4 ± 0.1
CVR (mmHg/ml/min/g)				
Vehicle	8.3 ± 0.3	3.1 ± 0.2	8.8 ± 0.7	8.4 ± 0.7
Acetaminophen	7.2 ± 0.3	3.1 ± 0.2	6.5 ± 0.5*	6.8 ± 0.4*

Note. Data are means ± SEM ($n = 10$). CPP, coronary perfusion pressure; CPF, coronary perfusate flow; CVR, coronary vascular resistance. Baseline, control conditions; 6 min hypoxia, 6 minutes low oxygen (5% O_2, 5% CO_2, balance N_2); 6 min reoxygenation, 6 minutes normoxia (95% O_2, 5% CO_2); 36 min reoxygenation, 36 minutes of normoxia.
* $P < 0.05$ relative to corresponding value for vehicle.

myocardial efficiency was improved during hypoxia and reoxygenation in acetaminophen-treated hearts.

At baseline, levels of myocardial efficiency were similar in the two treatment groups (1240 ± 134 and 1219 ± 248 mmHg/μl per gram in acetaminophen and vehicle-treated hearts, respectively). During hypoxia, acetaminophen-treated hearts maintained greater efficiency than vehicle-treated hearts (5052 ± 316 vs. 2183 ± 404 mmHg/μl per gram; $P < 0.05$); this was also observed during early reoxygenation [1265 ± 146 versus 793 ± 109 mmHg/μl/g ($P < 0.05$) in acetaminophen- and vehicle-treated hearts, respectively].

Metabolic Variables and Creatine Kinase Release. Not Po_2, pH, or base excess differed significantly between the two groups of hearts during baseline and reoxygenation (Table 2). There were small but significant differences in Pco_2 during hypoxia.

Creatine kinase activity did not differ between groups at baseline (Fig. 3). However, CK activity was significantly attenuated in acetaminophen- versus vehicle-treated hearts during hypoxia and reoxygenation. Hypoxic levels of CK activity were 2.9 ± 1.8 versus 9.7 ± 2.2 U/l ($P < 0.05$) in acetaminophen- and vehicle-treated hearts, respectively. During early reoxygenation, the mean CK activity for acetaminophen-treated hearts was 4.8 ± 0.9 U/l, compared with 83 ± 33 U/l for vehicle-treated hearts ($P < 0.05$).

Myofibrillar Ultrastructure. The appearance of myocardial tissue was similar in acetaminophen- (Fig. 4A) and vehicle-treated (Fig. 4B) samples during baseline. Contractile elements appeared normal, and mitochondria were similar in the two micrographs. After 6 min of hypoxia (Fig. 4C, acetaminophen treated; Fig. 4D, vehicle treated), the acetaminophen-treated sample retained the baseline appearance, whereas the vehicle-treated sample showed evidence of tissue damage (e.g., disrupted Z-lines). At 36 min reoxygenation, the micrograph of the acetaminophen-treated tissue (Fig. 4E) retained the characteristics seen in the baseline state, whereas the micrograph of the vehicle-treated sample (Fig. 4F) showed extensive tissue damage including swollen mitochondria and disrupted contractile elements.

Reactive Oxygen Species. As shown in Figure 5, coronary venous effluent samples from acetaminophen-treated hearts significantly attenuated peroxynitrite-mediated chemiluminescence compared to effluent from matched vehicle-treated hearts. For example, the average percentage inhibition of control during early reoxygenation was 56 ± 10% versus 99 ± 1% ($P < 0.05$) in vehicle- and acetaminophen-treated hearts, respectively. The potential of coronary venous effluent samples from the two

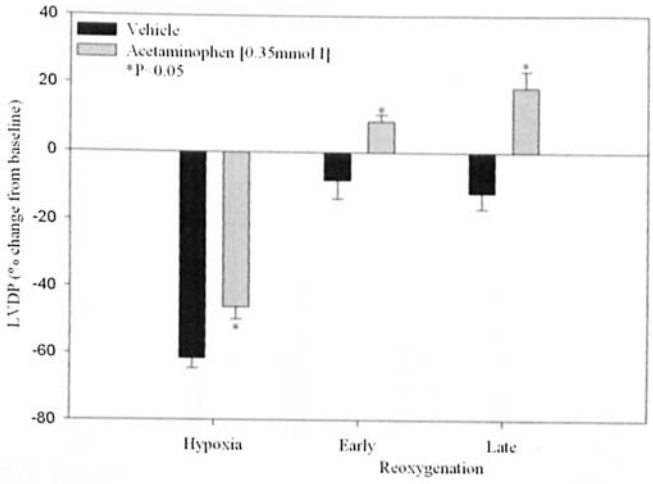

Figure 1. Influence of hypoxia and reoxygenation on left ventricular developed pressure (LVDP; percentage change from baseline) in acetaminophen- (0.35 m*M*) and vehicle-treated hearts. Note the significantly improved LVDP at hypoxia and both early and late reoxygenation in acetaminophen-treated hearts. Baseline values for LVDP were 41 ± 4 and 33 ± 2 mmHg in acetaminophen- and vehicle-treated hearts, respectively (not significantly different). Hypoxia, 6 min low oxygen (5%O_2, 5%CO_2, balance nitrogen); early reoxygenation, 6 min normoxia (95%O_2, 5%CO_2); late reoxygenation, 36 min normoxia. * $P < 0.05$ relative to corresponding vehicle mean; vertical bars represent the standard error.

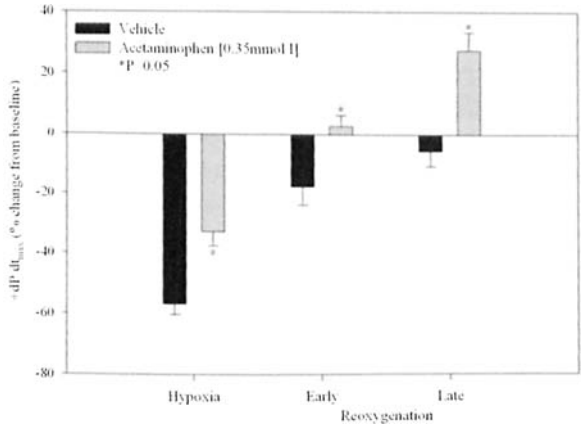

Figure 2. Influence of hypoxia and reoxygenation on the positive differentiation of left ventricular developed pressure ($+dP/dt_{max}$; percentage change from baseline). Note that this variable is improved during hypoxia and throughout the duration of reoxygenation in acetaminophen- (0.35 m*M*) versus vehicle-treated hearts. Baseline values for $+dP/dt_{max}$ were 1115 ± 147 and 989 ± 75 mmHg/s in acetaminophen- and vehicle-treated hearts, respectively (not significantly different). Hypoxia, 6 min low oxygen (5%O_2, 5%CO_2, balance nitrogen); early reoxygenation, 6 min normoxia (95%O_2, 5%CO_2); late reoxygenation, 36 min normoxia. * $P < 0.05$ relative to corresponding vehicle mean; vertical bars represent the standard error.

treatment groups to inhibit superoxide-mediated chemiluminescence was not significantly different, as both treatments showed near uniform inhibition of chemiluminescence (approximately 85 ± 5%; Fig. 6). Coronary effluent from acetaminophen-treated hearts contained quantitatively more nitrite than vehicle-treated hearts, although significance was not reached at any recorded time interval (data not shown).

Discussion

The effects of hypoxia and reoxygenation on the mammalian myocardium are well known (23). Hypoxic hearts experience reduced contractility, increased incidence of arrhythmias, and severe cellular damage (24). These effects are attributed to the production of damaging reactive oxygen species (9, 25).

Under conditions of ischemia and reperfusion, acetaminophen treatment results in significant improvement of left ventricular function as compared to control hearts (2–6). This cardioprotection is mediated by the ability of acetaminophen to reduce damaging oxygen and nitrogen radicals (4, 5). Investigating acetaminophen in hypoxia/reoxygenation would shed further light on its cardioprotective efficacy, as ischemia/reperfusion contains an intrinsic hypoxia/reoxygenation component. We therefore hypothesized that acetaminophen would be protective in hypoxia and reoxygenation via the attenuation of damaging reactive oxygen species, similar to the protection observed in ischemia and reperfusion.

In this study, acetaminophen-treated hearts retained a greater fraction of ventricular function during hypoxia and reoxygenation than did vehicle-treated hearts. In addition, coronary venous effluent from acetaminophen-treated hearts reduced damaging reactive oxygen species. Therefore, the acetaminophen-mediated cardioprotection seen during hypoxia and reoxygenation is similar to that observed during ischemia and reperfusion.

Hemodynamic and Mechanical Effects. The injurious effects of hypoxia and reoxygenation on left ventricular function were attenuated by acetaminophen. The early period of reoxygenation is associated with the

Table 2. Influence of Hypoxia and Reoxygenation with or without Acetaminophen Treatment on the Metabolic Status of the Isolated Guinea Pig Heart as a Function of Time

	Baseline		6 min Hypoxia		6 min Reoxygenation		36 min Reoxygenation	
	Acet	Vehicle	Acet	Vehicle	Acet	Vehicle	Acet	Vehicle
Po_2 (mmHg)								
Arterial	557 ± 18	530 ± 23	60 ± 3	55 ± 2	548 ± 12	556 ± 13	576 ± 15	570 ± 18
Venous	175 ± 14	173 ± 11	31 ± 2	31 ± 2	227 ± 20	219±19	163 ± 15	184 ± 17
Pco_2 (mmHg)								
Arterial	34 ± 1	34 ± 1	32 ± 1	32 ± 1	35 ± 1	34 ± 1	34 ± 1	35 ± 1
Venous	48 ± 1	46 ± 1	46 ± 1*	43 ± 1	48 ± 1	46 ± 1	50 ± 1	48 ± 1
pH								
Arterial	7.41 ± 0.01	7.40 ± 0.01	7.42 ± 0.01	7.42 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.40 ± 0.01	7.39 ± 0.01
Venous	7.28 ± 0.01	7.30 ± 0.01	7.28 ± 0.01	7.30 ± 0.01	7.28 ± 0.01	7.30 ± 0.01	7.26 ± 0.01	7.27 ± 0.01
Base Excess (mmol/l)								
Arterial	−3.3 ± 0.3	−4.0 ± 0.3	−3.9 ± 0.4	−4.2 ± 0.2	−3.8 ± 0.4	−4.5 ± 0.5	−3.7 ± 0.2	−4.3 ± 0.4
Venous	−4.7 ± 0.3	−4.6 ± 0.3	−5.6 ± 0.4	−5.6 ± 0.3	−4.9 ± 0.4	−4.8 ± 0.4	−5.1 ± 0.3	−5.0 ± 0.4
MVo_2 (µl/min/g)	64 ± 5	57 ± 5	12 ± 1	11 ± 2	60 ± 5	52 ± 4	73 ± 4	61 ± 5

Note. Data are means ± SEM ($n = 10$). Baseline, control conditions; 6 min hypoxia, 6 minutes low oxygen (5% O_2, 5% CO_2, balance N_2); 6 min reoxygenation, 6 minutes normoxia (95% O_2, 5% CO_2); 36 min reoxygenation, 36 minutes of normoxia. Acet, acetaminophen treated hearts; Vehicle, vehicle treated hearts; Po_2, partial pressure of oxygen; Pco_2, partial pressure of carbon dioxide; MVo_2, calculated myocardial oxygen consumption. Acetaminophen was added to the perfusate reservoir at a concentration of 0.35 m*M*.
* $P < 0.05$ relative to corresponding value for vehicle.

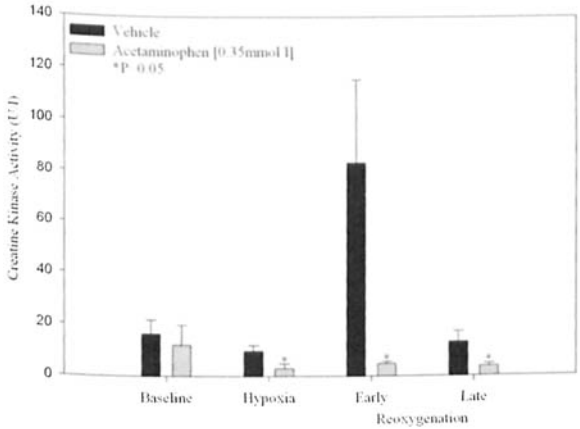

Figure 3. Influence of hypoxia and reoxygenation on creatine kinase (CK) activity (U/L). Note that CK activity is significantly reduced in acetaminophen-treated (0.35 m*M*) hearts versus vehicle-treated hearts throughout the duration of hypoxia and reoxygenation. Baseline, control conditions; hypoxia, 6 min low oxygen ($5\%O_2$, $5\%CO_2$, balance nitrogen); early reoxygenation, 6 min normoxia ($95\%O_2$, $5\%CO_2$); late reoxygenation, 36 min normoxia. * $P < 0.05$ relative to corresponding vehicle mean; vertical bars represent the standard error.

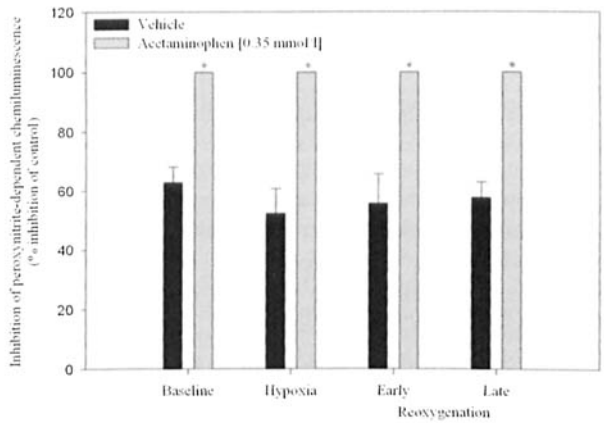

Figure 5. Effect of coronary effluent samples on peroxynitrite-mediated luminol oxidation. Note that the acetaminophen-treated (0.35 m*M*) samples inhibit chemiluminescence to a greater degree than the vehicle-treated samples at all recorded time intervals. Baseline, control conditions; hypoxia, 6 min low oxygen ($5\%O_2$, $5\%CO_2$, balance nitrogen); early reoxygenation, 3 min normoxia ($95\%O_2$, $5\%CO_2$); late reoxygenation, 36 min normoxia. * $P < 0.05$ relative to corresponding vehicle mean; vertical bars represent the standard error.

"oxygen paradox." Similar to early reperfusion, this period is associated with a burst of reactive oxygen species and a parallel reduction in ventricular mechanical function (26). We have previously shown that acetaminophen attenuates

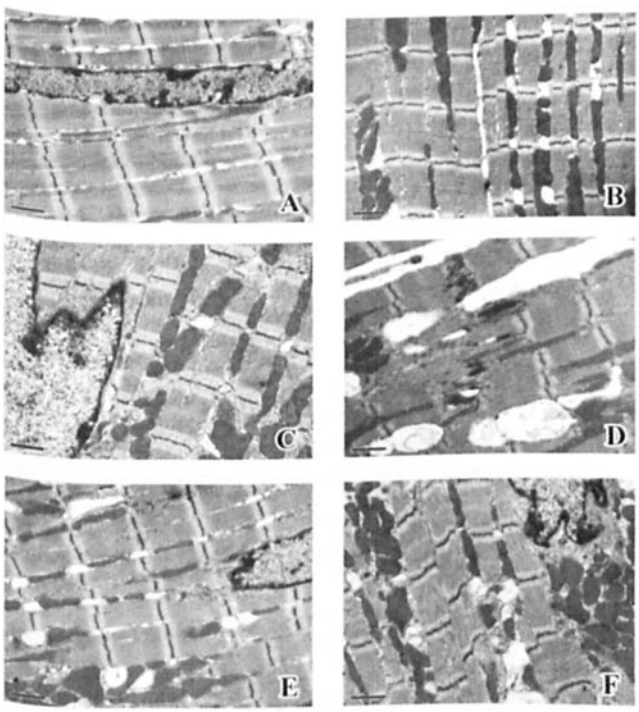

Figure 4. Electron micrographs of acetaminophen-treated (A, C, E) and vehicle-treated (B, D, F) tissue samples. Samples were taken at baseline (A, B), after 6 min of hypoxia ($5\%O_2$, $5\%CO_2$, balance nitrogen) (C, D), and after 6 min of hypoxia followed by 36 min of normoxic reoxygenation ($95\%O_2$, $5\%CO_2$) (E, F). Note the similarity between acetaminophen- and vehicle-treated samples at baseline, and the ill-defined ultrastructure (e.g., light and dark bands) in the presence of vehicle during hypoxia and reoxygenation. All scale bars equal 1 μm.

damaging oxygen radicals during the early stages of postischemia reperfusion (4–6). From the current results, it appears that acetaminophen is also able to reduce the tissue oxidant load during the early stages of posthypoxia reoxygenation, and thus preserve mechanical function (although no concomitant increase in MVO_2 was noted). The improvement is likely a result of the phenolic structure and antioxidant capacity of the drug. Data indicate that acetaminophen can attenuate the formation of peroxynitrite in the myocardium, which is known to damage myocytes through lipid peroxidation and therefore contribute to decreased contractility (27). In addition to preserving contractility during reoxygenation, acetaminophen also improved mechanical variables during hypoxia, a period

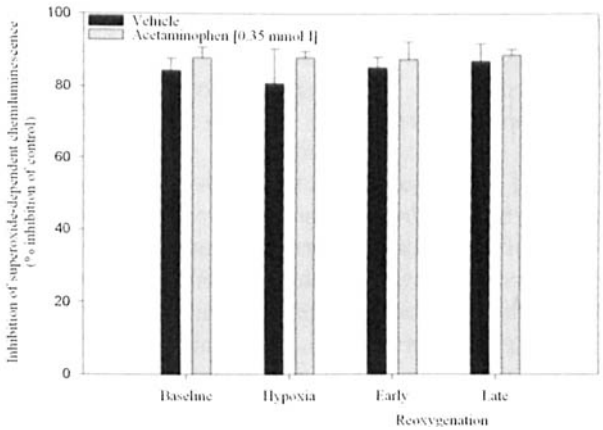

Figure 6. Effect of coronary effluent samples on superoxide-mediated lucigenin oxidation. Note the lack of significant difference in both acetaminophen (0.35 m*M*) and vehicle treatments. Baseline, control conditions; hypoxia, 6 min low oxygen ($5\%O_2$, $5\%CO_2$, balance nitrogen); early reoxygenation, 3 min normoxia ($95\%O_2$, $5\%CO_2$); late reoxygenation, 36 min normoxia. Vertical bars represent the standard error.

of relatively low oxidant stress. The mechanism for this improvement is unknown.

Although a trend toward greater nitrite content in acetaminophen-treated hearts was noted, total nitrite content did not reach significance between treatment groups, and the mechanism for the significant decrease in CVR in acetaminophen-treated hearts remains unknown.

Effects on Metabolic Variables and Creatine Kinase Release. Partial pressures of arterial oxygen (Po_2) around 550 mmHg are standard in this experimental setting, and MVo_2 homeostasis is maintained in the normoxic state around 60 μl/min per gram, as previously reported (28). The reduced partial pressure of oxygen and an overall decrease in MVo_2 in the hypoxic state reflect previous studies in Langendorff perfused hearts (10, 11, 28).

Overall, the metabolic data indicate that the hearts were stable and functioning normally in the experimental setting. The data also confirm that acetaminophen treatment had no effect on the basal metabolic status of the isolated guinea pig heart.

Creatine kinase is a reliable marker of myocardial tissue damage, and investigators often use CK to show cardioprotective efficacy of different compounds (29). Although the levels of CK associated with histologic damage are not well defined, the release of CK requires a leaky plasma membrane and degradation of subcellular structure; thus, quantitatively greater amounts of CK are associated with increased myocardial damage (29). Acetaminophen effectively attenuates CK activity in the ischemic and reperfused myocardium (2, 3), and this study shows attenuation of CK activity by acetaminophen in hypoxia and reoxygenation. Thus acetaminophen attenuates whatever cellular/molecular mechanisms are involved in elaborating CK during hypoxia and reoxygenation.

Reactive Oxygen Species. Van Dyke *et al.* (20) found that acetaminophen is a potent inhibitor of peroxynitrite-mediated chemiluminescence, and Merrill (4) found that coronary effluent samples from acetaminophen-treated hearts exposed to ischemia and reperfusion inhibit peroxynitrite production. In this study, coronary effluent from acetaminophen-treated hearts exhibits similar efficacy in inhibition of peroxynitrite-mediated chemiluminescence. Therefore, the antioxidant capacity of acetaminophen is similar in ischemia/reperfusion and hypoxia/reoxygenation.

The combination of superoxide and nitric oxide is the most common pathway for the production of peroxynitrite (30). Although acetaminophen retains its antioxidant capacity against peroxynitrite, it does not have concomitant effects against superoxide or nitric oxide. Therefore, acetaminophen is able to directly reduce native peroxynitrite, and not its most common radical components.

Myofibrillar Ultrastructure. The preservation of myofibrillar ultrastructure with acetaminophen treatment has been demonstrated in an ischemia/reperfusion setting (2, 3). The results of this experiment are consistent with those obtained from ischemia and reperfusion. The tissue damage observed in vehicle-treated samples is consistent with other data obtained in this study (e.g., preservation of mechanical function, attenuation of creatine kinase activity) and provides evidence of cardioprotection with acetaminophen. Because of the limited number of hearts analyzed, however, these data are suggestive.

Limitations. Although the use of the isolated Langendorff perfused guinea pig heart offers many advantages to *in vivo* preparations, it may also have significant limitations. The elimination of complicating factors is a clear advantage to the preparation. However, our laboratory uses a crystalloid perfusate, as opposed to a whole-blood perfusate with colloid and cellular elements. These cellular elements may be vital to the removal of damaging oxidants, as well as to oxygen delivery. In addition, the use of guinea pigs in this study may also be confounded by their inability to produce ascorbic acid, a first-line defense against reactive oxygen species (31). The perfusate reservoir also does not contain ascorbic acid, and a comparison of the antioxidative capabilities of ascorbic acid and acetaminophen is warranted in further investigations. Although the utility of artificial perfusates and guinea pig models are still under debate (32, 33), the Langendorff preparation remains a proven system for the determination of myocardial function.

Summary and Conclusions

In the isolated guinea pig heart exposed to hypoxia and reoxygenation, acetaminophen is cardioprotective. This is evident in the preservation of contractile function, in the maintenance of myofibrillar integrity, and in the attenuation of damaging cardiac markers. The preservation of myocardial contractility by acetaminophen may be mediated by the antioxidant nature of the drug, as coronary effluent from acetaminophen-treated hearts reduces damaging reactive oxygen species.

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