Attenuation of Cardiac Stunning by Losartan in a Cellular Model of Ischemia and Reperfusion Is Accompanied by Increased Sarcoplasmic Reticulum Ca\textsuperscript{2+} Stores and Prevention of Cytosolic Ca\textsuperscript{2+} Elevation

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Received June 14, 2004; accepted August 9, 2004

ABSTRACT

This study investigates whether protective effects of an angiotensin II type 1 receptor antagonist (losartan) in ischemia and reperfusion are mediated by actions on Ca\textsuperscript{2+} cycling. Effects of exposure to losartan (10 \mu M) in ischemia were evaluated in isolated guinea pig ventricular myocytes exposed to simulated ischemia and reperfusion at 37°C. Field-stimulated myocytes were exposed to 30 min of simulated ischemia (hypoxia, acidosis, lactate, hyperkalemia, and glucose-free) and reperfusion with Tyrode’s solution for 40 min. Cell shortening was measured with a video edge detector, and Ca\textsuperscript{2+} concentration was measured with fura-2. Field-stimulated myocytes exhibited stunning in reperfusion, which was abolished in cells exposed to losartan. In microelectrode studies, losartan did not alter the responses of resting potentials or action potentials to ischemia and reperfusion. In the absence of losartan, diastolic Ca\textsuperscript{2+} increased in ischemia, and Ca\textsuperscript{2+} transients exhibited a rebound overshoot in early reperfusion. Losartan did not affect amplitudes of Ca\textsuperscript{2+} transients in ischemia but prevented elevations in diastolic Ca\textsuperscript{2+} in ischemia. Furthermore, losartan prevented the overshoot of Ca\textsuperscript{2+} transients in early reperfusion and increased the magnitude of Ca\textsuperscript{2+} transients in late reperfusion. Sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} stores, determined as Ca\textsuperscript{2+} released by rapid application of 10 mM caffeine, were not altered in ischemia and reperfusion. However, losartan increased SR Ca\textsuperscript{2+} stores in late reperfusion, even in cells that were not exposed to simulated ischemia. We conclude that losartan abolishes stunning in reperfusion by preserving normal diastolic Ca\textsuperscript{2+} in ischemia and by increasing Ca\textsuperscript{2+} transients through elevation of releasable SR Ca\textsuperscript{2+}.

Previous studies have suggested that activation of the renin-angiotensin system may contribute to deleterious effects of myocardial ischemia and reperfusion (for review, see Ertl and Hu, 2001). Production of angiotensin II increases during myocardial ischemia, and levels of circulating angiotensin II rise (Ertl and Hu, 2001). Furthermore, angiotensin-converting enzyme (ACE) inhibitors, which inhibit the production of angiotensin II, exert protective effects in myocardial ischemia (for review, see Pfeffer, 2000). ACE inhibitors have been shown to attenuate postischemic contractile depression or stunning and to suppress arrhythmias in ischemia and reperfusion (Przyklenk and Kloner, 1987; Westlin and Mullane, 1988; Ehring et al., 1994; Nakai et al., 1999; Zhu et al., 2000).

However, ACE inhibitors not only decrease production of angiotensin II, they also promote accumulation of the potent vasodilator bradykinin (Ehring et al., 1994). Thus, both reduction in angiotensin II production and increased bradykinin-mediated vasodilation may contribute to protective effects of ACE inhibitors in myocardial ischemia.

A number of studies have demonstrated that blockade of angiotensin II receptors with selective angiotensin II type 1 (AT\textsubscript{1}) receptor antagonists also is protective in myocardial ischemia and reperfusion (for review, see Schulz and Heusch, 2003). Pretreatment with the AT\textsubscript{1} receptor antagonists losartan or candesartan improves recovery of function and inhibits arrhythmias in Langendorff-perfused hearts exposed to global ischemia (Lee et al., 1997; Paz et al., 1998; Wang and Sjoquist, 1999). Similar protective effects of AT\textsubscript{1} antagonists have been reported in in vivo models subjected to transient coronary artery ligation (Harada et al., 1998; Dorge et al., 1999; Zhu et al., 2000; Yahiro et al., 2003). Other studies, however, in intact hearts have reported that AT\textsubscript{1} receptor

ABBREVIATIONS: ACE, angiotensin-converting enzyme; AT\textsubscript{1} receptor, angiotensin II type 1 receptor; EXP3174, 2-n-butyl-4-chloro-1-[(2‘-([1H]-tetrazol-5-yl)-biphenyl-4-yl)methyl]imidazole-5-carboxylic acid; SR, sarcoplasmic reticulum; APD, action potential duration; RMP, resting membrane potential.
antagonists actually have deleterious effects on recovery of contractile function in reperfusion (Ford et al., 1996, 1998; So et al., 1998). Thus, whether AT1 receptor antagonists exert protective effects in intact hearts exposed to ischemia and reperfusion remains controversial.

We recently reported that losartan attenuates stunning and inhibits the arrhythmogenic transient inward current in voltage-clamp studies of isolated ventricular myocytes exposed to simulated ischemia and reperfusion (Louch et al., 2000). Interestingly, losartan exerted these protective effects both in the absence and presence of exogenous angiotensin II, which suggests that losartan has effects on cardiac myocytes that are independent of AT1 receptor blockade (Louch et al., 2000). Elevations in intracellular free Ca2+ are believed to promote stunning (for review, see Bolli and Marban, 1999) and induce transient inward current (Lederer and Tsien, 1976). Therefore, it is possible that the protective effects of losartan in ischemia and reperfusion are mediated by alterations in Ca2+ cycling and a reduction in intracellular free Ca2+ levels.

Our previous investigation of losartan in ischemia and reperfusion was conducted under voltage-clamp conditions to correlate effects on L-type Ca2+ current and contraction (Louch et al., 2000). However, changes in action potential configuration in response to ischemia and reperfusion would be predicted to contribute to alterations in Ca2+ cycling and contractile responses in ischemia and reperfusion. Therefore, the goal of the present study was to examine the effects of losartan on Ca2+ cycling and stunning in field or intracellularly stimulated cardiac myocytes where changes in action potential configuration are not eliminated by voltage clamp. Experiments were conducted in a cellular model of simulated ischemia and reperfusion developed in this laboratory (Cordeiro et al., 1994; Louch et al., 2000, 2002). Isolated myocytes exposed to simulated ischemia exhibit many characteristics of true ischemia such as depolarization, abbreviation of action potential duration, intracellular Ca2+ overload, and abolition of contraction (Cordeiro et al., 1994; Louch et al., 2000, 2002). Myocytes also exhibit the arrhythmogenic transient inward current in early reperfusion and stunning in late reperfusion (Cordeiro et al., 1994; Louch et al., 2000, 2002). The specific objectives of this study were to determine whether losartan: 1) attenuates stunning in field-stimulated guinea pig ventricular myocytes exposed to simulated ischemia and reperfusion; 2) alters resting or action potentials in myocytes subjected to ischemia and reperfusion; 3) prevents elevation of intracellular free Ca2+ levels in myocytes exposed to ischemia and reperfusion; and 4) alters Ca2+ transients and/or sarcoplasmic reticulum (SR) Ca2+ load in cells exposed to simulated ischemia and reperfusion and under normoxic conditions.

Materials and Methods

Myocyte Isolation. All experiments were conducted on freshly isolated guinea pig ventricular myocytes and performed in accordance with guidelines published by the Canadian Council on Animal Care. Albino guinea pigs (250-400 g; Charles River Canada, St. Constant, QC, Canada), of which approximately 90% were male and 10% female, were used in this investigation. Guinea pigs were injected with heparin (3.3 IU/g) and anesthetized with sodium pentobarbital (160 mg/kg). The heart was then rapidly cannulated and perfused retrogradely through the aorta (10–12 ml/min) for 7 to 8 min with oxygenated (100% O2; 37°C) Ca2+-free solution of the following composition: 120 mM NaCl, 3.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 10 mM HEPES, and 11 mM glucose (pH 7.4 with NaOH). Collagenase A (25 mg/50 ml) and protease (4.8 mg/50 ml, Sigma-type XIV) were then included in the perfusate for approximately 5 min. Following dissociation, the ventricles were minced and washed in a substrate-enriched high K+ buffer of the following composition: 80 mM KOH, 50 mM glutamate acid, 30 mM KCl, 30 mM KH2PO4, 20 mM taurine, 10 mM HEPES, 10 mM glucose, 3 mM MgSO4, 0.5 mM EGTA (pH 7.4 with KOH). This isolation procedure provided ventricular myocytes that were rod-shaped and free of membrane blebs. Myocytes were then placed in a 0.75-ml chamber on the stage of an inverted microscope. Cells were allowed to adhere to the bottom of the chamber for 5 to 10 min and then were superfused (3 ml min−1, 37°C) with normal Tyrode’s solution of the following composition: 129 mM NaCl, 20 mM NaHCO3, 0.9 mM NaH2PO4, 4 mM KCl, 0.5 mM MgSO4, 2.5 mM CaCl2, and 5.5 mM glucose, pH 7.4, gassed with 95% O2, 5% CO2.

General Methods. Cells that served as time controls were exposed to 80 min of superfusion with normal Tyrode’s solution. In all other experiments, myocytes were exposed to 10 min of normal Tyrode’s solution and were then superfused for 30 min with an “ischemic” Tyrode’s solution. The ischemic solution was designed to mimic conditions associated with ischemia including hypoxia, hypercapnia, hyperkalemia, acidosis, lactate accumulation, and substrate deprivation (Ferrier and Guyette, 1991). This solution had the following composition: 123 mM NaCl, 6 mM NaHCO3, 0.9 mM NaH2PO4, 8 mM KCl, 0.5 mM MgSO4, 2.5 mM CaCl2, and 20 mM sodium lactate, gassed with 90% N2/10% CO2 gas phase was layered over the superfusion chamber throughout simulated ischemia. Reperfusion was achieved by superfusion with normal Tyrode’s solution. Each cell was exposed to only one cycle of ischemia and reperfusion. Cells in the treatment group were exposed to 10 μM losartan only during ischemia. This concentration was chosen on the basis of a previous study that reported that 10 μM but not 1.0 μM losartan exerted protective effects in ischemia and reperfusion and that 10 μM losartan blocked the effects of exogenous angiotensin II in guinea pig ventricular tissue (Thomas et al., 1996).

Measurement of Contractions. In some experiments, myocytes were field-stimulated continuously at 2 Hz through a pair of platinum electrodes. Myocytes were visualized with a video camera and TV monitor. Cell length was recorded with a video edge detector (Crescent Electronics, Windsor, ON, Canada). Contraction was measured as the maximum difference between diastolic and systolic cell length. Cell length was recorded at 5-min intervals throughout the experiment, except during the first 5 min of reperfusion when recordings were made every minute. Three responses were averaged for each recording period.

Measurement of Action Potentials. In other experiments, action potentials were measured with conventional microelectrode techniques. Cells were impaled with high-resistance electrodes (18–25 MΩ) to minimize dialysis and avoid buffering intracellular Ca2+ levels. Electrodes were filled with 2.7 M KCl, and a 2.7 M KCl-agar bridge was used as a bath ground. Myocytes were stimulated by 3.5-ms current pulses delivered through the recording electrode. Action potentials (averages of five) were recorded every 5 min. Cells were activated regularly throughout the experiments. Recordings were made with an Axoclamp 2B amplifier (Axon Instruments, Inc., Union City, CA). Resting membrane potential (RMP) was measured just prior to action potential trains. Action potential duration (APD) was measured at 80% repolarization with respect to the action potential amplitude.

Intracellular Ca2+ Measurements. Intracellular Ca2+ was measured by whole cell photometry (DeltaRam; Photon Technology International, Monmouth Junction, NJ). Myocytes were loaded with fura-2 by incubation with fura-2/acetoxyethyl ester (0.1 μM) for 20 min in the dark at room temperature. The ratio of emission at 510 nm, during alternate excitation at 340 and 380 nm, was used to

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determine intracellular Ca\(^{2+}\) concentrations. The fluorescence ratio was converted to Ca\(^{2+}\) concentration with a calibration curve determined experimentally at pH 7.2 as described previously (Louch et al., 2002). Ca\(^{2+}\) concentration measured with this calibration curve is expected to slightly underestimate actual intracellular Ca\(^{2+}\) during ischemia since intracellular acidosis increases the dissociation constant of fura-2 (Martinez-Zaguilan et al., 1991). However, this is not likely to be a problem in reperfusion as intracellular pH recovers rapidly to preischemic levels upon reperfusion (Kitakaze et al., 1988). Fluorescence was recorded and measured with Felix software (version 1.4; Photon Technology International).

Ca\(^{2+}\) transients were recorded in both field-stimulated myocytes and in cells stimulated through the microelectrode. Recordings were background-subtracted, and the magnitude of the Ca\(^{2+}\) transient was calculated as the difference between diastolic and peak systolic Ca\(^{2+}\) concentrations. Ca\(^{2+}\) transients were recorded at 5-min intervals throughout the experiment, except for the first 5 min of reperfusion when recordings were made every minute. Three responses were averaged for each recording period.

**Estimation of SR Ca\(^{2+}\) Stores.** Amplitudes of caffeine-elicited Ca\(^{2+}\) transients were used as a measure of SR Ca\(^{2+}\) content. In these experiments, cells were stimulated with current pulses delivered through the microelectrode at 2 Hz. At 10, 40, 55, 70, and 80 min during the experiment, stimulation was briefly interrupted, and 10 mM caffeine was applied to cells for 1 s at 37°C with a rapid solution switcher. The rapid solution switcher was triggered by the voltage-clamp protocol and changed the solution bathing the cell within 300 ms.

**Analyses.** Differences between experimental groups were tested for statistical significance with a two-way repeated measures analysis of variance. All other data were analyzed relative to preischemic values with a one-way repeated measures analysis of variance. Post hoc comparisons were made with a Bonferroni test. Statistical analyses were performed with Sigma Stat (version 2.0; SPSS Science, Chicago, IL). Data are presented as means ± S.E.M. The value of “n” represents the number of myocytes sampled (no more than 3 myocytes from a single heart were used).

**Compounds.** Losartan was a gift from Merck Frost Canada Inc. (Kirkland, QC, Canada). Collagenase A was obtained from Roche Diagnostics (Laval, QC, Canada). Fura-2-acetoxymethyl ester was purchased from Invitrogen Canada Inc. (Burlington, ON, Canada). All other drugs and chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

**Results**

**Effects of Losartan on Contractions throughout Ischemia and Reperfusion.** Figure 1 shows representative recordings of cell length at selected time points during ischemia and reperfusion. Control recordings in the absence of drug are shown in Fig. 1A, and recordings obtained in a cell exposed to 10 μM losartan in ischemia are shown in Fig. 1B. In the absence of losartan, contractions were markedly reduced in ischemia, and diastolic length increased slightly (Fig. 1A). After 1 min of reperfusion, contractions recovered and exceeded preischemic levels, and diastolic length decreased (Fig. 1A). After 10 min of reperfusion, diastolic cell length recovered slightly, but contractions were markedly depressed relative to preischemic levels (Fig. 1A). Similar results were obtained in ischemia and early reperfusion in the cell exposed to losartan (Fig. 1B); however, losartan improved the recovery of contractions at 10 min of reperfusion (Fig. 1B).

Figure 2A shows mean measurements of cell shortening during time-control experiments and during exposure to simulated ischemia and reperfusion. In time-control experiments, myocytes exhibited only a slight reduction in cell shortening during 80 min of recording. During ischemia, myocytes exhibited a significant decrease in contractions relative to time controls (Fig. 2A). In early reperfusion, contractions were briefly but significantly increased from time-control values. However, after the first few minutes of reperfusion, myocytes exhibited a significant reduction in cell shortening for the remainder of the protocol (Fig. 2A). Losartan-treated cells did not differ significantly from time-control values. However, after the first few minutes of reperfusion, myocytes exhibited a significant reduction in cell shortening for the remainder of the protocol (Fig. 2A). Losartan-treated cells were similar to untreated cells during ischemia and early reperfusion (Fig. 2B); however, postischemic contractile depression was attenuated following losartan treatment (Fig. 2B). Indeed, cell shortening in losartan-treated cells did not differ significantly from time-control values at any point in reperfusion (Fig. 2B). Thus, field-stimulated myocytes exhibited stunning in reperfusion, which was attenuated by losartan treatment during ischemia.

**Effects of Losartan on Resting Membrane Potential and Action Potential Duration during Ischemia and Reperfusion.** Contractions in field-stimulated myocytes are initiated by action potentials. Thus, it is possible that losartan might have improved recovery of contractions in reperfusion by effects on RMP or APD. To determine whether losartan treatment altered electrical properties of cells, cells were impaled with microelectrodes. Action potentials were elicited by a 3.5-ms current stimulus delivered through the electrode. Figure 3A shows a representative recording of action potentials in a control cell at selected time points during ischemia and reperfusion. The cell depolarized, and action potentials abbreviated in ischemia; these changes recovered gradually in reperfusion (Fig. 3A). Similar results are shown in representative recordings from a cell exposed to losartan in ischemia (Fig. 3B).
Losartan Attenuates Ischemia/Reperfusion Injury

Fig. 2. Losartan protects against stunning in isolated cardiac myocytes. A, mean data demonstrate that ischemia caused a significant decrease in the amplitudes of contractions relative to time controls in the absence of losartan. Upon reperfusion, contractions initially increased; however, significant contractile depression (stunning) was observed with continued reperfusion. B, losartan-treated myocytes also exhibited a significant reduction in the magnitude of contractions in ischemia, followed by an increase in early reperfusion. However, stunning was attenuated in losartan-treated myocytes. Cell shortening was normalized as a percent of preischemic values. *p < 0.05 denotes significant difference from time controls; n = 15 myocytes from 10 hearts in the ischemia/reperfusion group; n = 18 myocytes from nine hearts in the losartan group; n = 12 myocytes from nine hearts in the time-control group.

Figure 4 shows mean measurements of RMP (panel A) and APD (panel B) during ischemia and reperfusion. In ischemia, control and losartan-treated cells depolarized to approximately −73 mV from preischemic values near −90 mV (Fig. 4A). RMP recovered rapidly upon reperfusion (Fig. 4A). Figure 4B shows that APD declined gradually during ischemia in both control and losartan-treated cells and recovered gradually in reperfusion in both groups. Thus, losartan treatment did not alter the changes in either RMP or APD during ischemia and reperfusion.

Effects of Losartan Treatment on Diastolic Ca^{2+} and Ca^{2+} Transients. In the next series of experiments, we determined whether losartan altered intracellular Ca^{2+} levels and Ca^{2+} transients in field-stimulated ventricular myocytes paced at 2 Hz. First, we examined intracellular Ca^{2+} levels and Ca^{2+} transients in time-control experiments where cells were not exposed to ischemia and reperfusion. Figure 5 shows representative recordings (panel A) and mean intracellular Ca^{2+} measurements (panel B) for time-control experiments. Ca^{2+} transient magnitudes were relatively stable for the duration of the protocol (80 min), although diastolic Ca^{2+} increased gradually with time (Fig. 5, A and B).

Next, we examined Ca^{2+} transients at selected time points throughout ischemia and reperfusion in the absence of losartan (Fig. 6, A and B). The representative example in Fig. 6A shows that diastolic Ca^{2+} increased in ischemia, but the magnitude of Ca^{2+} transients was unchanged relative to preischemic conditions. At 1 min of reperfusion, diastolic Ca^{2+} levels declined toward preischemic levels, and Ca^{2+} transients increased in magnitude (Fig. 6A). After 30 min of reperfusion, Ca^{2+} transients had recovered, and diastolic Ca^{2+} levels were increased (Fig. 6A). Figure 6B shows mean intracellular Ca^{2+} measurements at intervals throughout ischemia and reperfusion. Diastolic Ca^{2+} increased significantly during ischemia, recovered rapidly in early reperfusion, and increased in late reperfusion (Fig. 6B). Ca^{2+} transient amplitudes (shaded area) also increased in early reperfusion (Fig. 6B).

Figure 6, C and D, also shows Ca^{2+} transients at selected time points throughout ischemia and reperfusion in the presence of losartan in ischemia. The representative example in Fig. 6C shows that diastolic Ca^{2+} increased slightly relative to preischemic levels during ischemia and reperfusion. Ca^{2+} transients did not increase in magnitude in early reperfusion but appeared larger after 30 min of reperfusion (Fig. 6C). Figure 6D shows that in the presence of losartan, mean intracellular Ca^{2+} levels increased significantly during ischemia and late reperfusion when compared with preischemic levels. Ca^{2+} transients did not increase in early reperfusion but did increase in late reperfusion (Fig. 6D).

The preceding results describe changes in diastolic and systolic Ca^{2+} levels in treated and untreated myocytes relative to respective preischemic values. To evaluate the effects of losartan on the effects of ischemia and reperfusion, we compared responses relative to time controls. Figure 7A compares mean diastolic Ca^{2+} levels recorded at selected times...
in ischemia and reperfusion to diastolic Ca\(^{2+}\) levels at corresponding times in time controls. Ischemia caused a significant increase in diastolic Ca\(^{2+}\) levels relative to time controls. This increase recovered rapidly in reperfusion (Fig. 7A). In contrast, when myocytes were exposed to losartan in ischemia, the increase in diastolic Ca\(^{2+}\) during ischemia was prevented (Fig. 7B). Figure 8A compares mean amplitudes of Ca\(^{2+}\) transients at different time points in time controls and cells exposed to simulated ischemia and reperfusion. Throughout most of the experiment, Ca\(^{2+}\) transient amplitudes were similar in time controls and in cells exposed to ischemia and reperfusion (Fig. 8A). However, Ca\(^{2+}\) transients were significantly increased in amplitude in early reperfusion (Fig. 8A). Interestingly, exposure to losartan in ischemia prevented the increase in amplitudes of Ca\(^{2+}\) transients in early reperfusion but increased the magnitude of Ca\(^{2+}\) transients late in reperfusion (Fig. 8B). These comparisons demonstrate that losartan attenuates the increase in diastolic Ca\(^{2+}\) levels during ischemia, prevents the overshoot of Ca\(^{2+}\) transients in early reperfusion, and increases the magnitude of Ca\(^{2+}\) transients in late reperfusion.

**Effects of Losartan on SR Ca\(^{2+}\) Stores during Ischemia and Reperfusion.** We next conducted experiments to determine whether losartan alters SR Ca\(^{2+}\) stores in ischemia and reperfusion. SR Ca\(^{2+}\) stores were assessed by rapid application of 10 mM caffeine with a rapid solution switcher. Representative recordings of caffeine-elicited Ca\(^{2+}\) transients in the absence and presence of losartan in ischemia are shown in Fig. 9, A and B. Caffeine-elicited Ca\(^{2+}\) transients appeared to decline gradually with time in cells exposed to simulated ischemia and reperfusion (Fig. 9A). However, the magnitude of caffeine transients increased throughout ischemia and reperfusion in myocytes exposed to losartan in ischemia (Fig. 9B).

Figure 9C shows mean measurements of the magnitude of caffeine-elicited Ca\(^{2+}\) transients at selected intervals during an experiment. Caffeine-induced Ca\(^{2+}\) transients declined slightly with time in both time controls and in cells exposed to simulated ischemia and reperfusion (Fig. 9C). However, caffeine transients increased with time when cells were exposed to losartan during ischemia and this increase was significant after 30 min of reperfusion (Fig. 9C). These observations suggest that exposure to losartan in ischemia may increase SR Ca\(^{2+}\) stores during reperfusion.

**Effects of Losartan on SR Ca\(^{2+}\) Stores and Intracellular Ca\(^{2+}\) Concentrations under Normoxic Conditions.** Exposure to losartan in ischemia appeared to markedly increase SR Ca\(^{2+}\) stores in late reperfusion. Therefore, we investigated whether losartan also might increase SR Ca\(^{2+}\) stores when cells are exposed to drug under normoxic conditions. In these experiments, myocytes were exposed to 10 μM losartan for 30 min, followed by 40 min of washout.
Caffeine was applied at regular intervals to evaluate SR Ca\(^{2+}\)/H\(_{11001}\) stores. Figure 10A shows mean data which demonstrate that losartan increased the magnitude of caffeine-elicited Ca\(^{2+}\)/H\(_{11001}\) transients even in the absence of ischemia and reperfusion. SR Ca\(^{2+}\)/H\(_{11001}\) stores continued to increase, even after the drug was washed out. Therefore, losartan caused a persistent increase in SR Ca\(^{2+}\)/H\(_{11001}\) stores under normoxic conditions. Despite this increase in SR Ca\(^{2+}\)/H\(_{11001}\) stores, signs of Ca\(^{2+}\)/H\(_{11001}\) overload were not observed.

We next determined whether a 30-min exposure to losartan under normoxic conditions altered Ca\(^{2+}\)/H\(_{11001}\) transients and diastolic Ca\(^{2+}\)/H\(_{11001}\) levels. Figure 10B shows mean amplitudes of Ca\(^{2+}\)/H\(_{11001}\) transients plotted as a function of time in time controls and in losartan-treated myocytes. Exposure to losartan in ischemia prevented the increase in amplitudes of Ca\(^{2+}\)/H\(_{11001}\) transients in early reperfusion and increased the magnitude of Ca\(^{2+}\)/H\(_{11001}\) transients in late reperfusion. Diastolic Ca\(^{2+}\)/H\(_{11001}\) was significantly elevated in late reperfusion in losartan-treated cells. \(\ast, p < 0.05\) denotes significantly different from preischemic values, \(n = 15\) myocytes from 12 hearts in the ischemia/reperfusion group; \(n = 11\) myocytes from nine hearts in the losartan group.

Fig. 6. Losartan treatment during ischemia altered the effects of ischemia and reperfusion on Ca\(^{2+}\) homeostasis. A, representative recordings of Ca\(^{2+}\) transients from a cell exposed to simulated ischemia and reperfusion and mean data are presented in B. Diastolic Ca\(^{2+}\) levels increased in ischemia with little change in the amplitude of Ca\(^{2+}\) transients (shaded region). Ca\(^{2+}\) transients temporarily exceeded preischemic levels in early reperfusion. Diastolic Ca\(^{2+}\) levels recovered in early reperfusion but rose again in late reperfusion. Representative recordings (C) and mean data (D) in cells treated with losartan show that diastolic Ca\(^{2+}\) increased during ischemia with no change in the magnitude of Ca\(^{2+}\) transients. However, exposure to losartan in ischemia prevented the increase in amplitudes of Ca\(^{2+}\) transients in early reperfusion and increased the magnitude of Ca\(^{2+}\) transients in late reperfusion. Diastolic Ca\(^{2+}\) was significantly elevated in late reperfusion in losartan-treated cells. \(\ast, p < 0.05\) denotes significantly different from preischemic values, \(n = 15\) myocytes from 12 hearts in the absence of losartan and 11 myocytes from nine hearts in the presence of losartan.

Caffeine was applied at regular intervals to evaluate SR Ca\(^{2+}\) stores. Figure 10A shows mean data which demonstrate that losartan increased the magnitude of caffeine-elicited Ca\(^{2+}\) transients even in the absence of ischemia and reperfusion. SR Ca\(^{2+}\) stores continued to increase, even after the drug was washed out. Therefore, losartan caused a persistent increase in SR Ca\(^{2+}\) stores under normoxic conditions. Despite this increase in SR Ca\(^{2+}\) stores, signs of Ca\(^{2+}\) overload were not observed.

We next determined whether a 30-min exposure to losartan under normoxic conditions altered Ca\(^{2+}\) transients and diastolic Ca\(^{2+}\) levels. Figure 10B shows mean amplitudes of Ca\(^{2+}\) transients plotted as a function of time in time controls and in losartan-treated myocytes. Exposure to losartan increased the amplitudes of Ca\(^{2+}\) transients after washout of the drug when compared with time controls. Figure 10C shows mean diastolic Ca\(^{2+}\) levels plotted as a function of time. Diastolic Ca\(^{2+}\) levels were not significantly different in losartan treated myocytes compared with time controls.

Thus, increased SR Ca\(^{2+}\) stores in myocytes exposed to losartan under normoxic conditions were accompanied by in-
creased amplitudes of Ca$^{2+}$/H$^{11001}$ transients without elevation of diastolic Ca$^{2+}$/H$^{11001}$ levels.

**Discussion**

The objectives of this study were to determine whether losartan: 1) attenuates stunning in field-stimulated guinea pig myocytes exposed to simulated ischemia and reperfusion, 2) alters resting or action potentials in ischemia and reperfusion, 3) prevents elevation of intracellular free Ca$^{2+}$/H$^{11001}$ levels in cells exposed to ischemia and reperfusion, and 4) alters Ca$^{2+}$/H$^{11001}$ transients and/or SR Ca$^{2+}$/H$^{11001}$ load in ischemia and reperfusion and under normoxic conditions. Our results show that losartan improved recovery of contractile function in field-stimulated myocytes following exposure to ischemia and reperfusion. Losartan did not alter the effects of ischemia or reperfusion on RMP and APD. Losartan prevented the elevation of diastolic Ca$^{2+}$/H$^{11001}$ levels in ischemia and abolished the overshoot of Ca$^{2+}$/H$^{11001}$ transients in early reperfusion. In addition, losartan increased SR Ca$^{2+}$/H$^{11001}$ stores even in cells that were not exposed to ischemia and reperfusion. Thus, losartan may improve contractile function in reperfusion by increasing SR Ca$^{2+}$/H$^{11001}$ stores and thereby SR Ca$^{2+}$/H$^{11001}$ release.
In the present study, we found that losartan reduced postischemic contractile depression in field-stimulated ventricular myocytes. Field-stimulated myocytes, contractions are activated by action potentials with configurations that change with ischemia and reperfusion (Cordeiro et al., 1994). However, we found that losartan did not alter changes in APD and RMP occurring in response to ischemia and reperfusion. Therefore, attenuation of stunning by losartan must occur through an action that does not involve alterations in APD or RMP. This interpretation is supported by our previous observation that exposure to losartan in ischemia improved recovery of contractile function in reperfusion when the magnitude and duration of depolarization was controlled with voltage clamp (Louch et al., 2000).

We also found that exposure to losartan during ischemia prevented elevation of diastolic Ca$^{2+}$ levels which typically occurs in response to ischemia. Prevention of elevated intracellular free Ca$^{2+}$ in ischemia may contribute to the protective effects of losartan in ischemia and reperfusion. Ca$^{2+}$ overload with elevation of cytosolic free Ca$^{2+}$ is believed to promote myocardial stunning by triggering Ca$^{2+}$-dependent proteolysis of myofilaments, which reduces myofilament responsiveness to Ca$^{2+}$ (Gao et al., 1996; Bolli and Marban, 1999). Thus, a reduction in intracellular free Ca$^{2+}$ by losartan in ischemia might preserve myofilament sensitivity and contribute to the attenuation of stunning observed in reperfusion.

Previous studies have shown that losartan decreases the incidence of ischemia and reperfusion-induced arrhythmias in in vivo mouse and rat models (Lee et al., 1997; Harada et al., 1998; Zhu et al., 2000). Furthermore, we previously have demonstrated an antiarrhythmic effect of losartan in early reperfusion in isolated cardiac ventricular muscle preparations exposed to simulated ischemia and reperfusion (Thomas et al., 1996). A reduction in intracellular Ca$^{2+}$ overload also could contribute to the antiarrhythmic actions of losartan reported in these studies. Intracellular Ca$^{2+}$ overload is believed to play an important role in the generation of reentrant arrhythmias (Thomas et al., 1996). In addition, Ca$^{2+}$ overload promotes the arrhythmogenic transient inward current, which is believed to cause triggered arrhythmias (Ferrier, 1977). Indeed, losartan has been shown to inhibit the arrhythmogenic transient inward current in early reperfusion (Louch et al., 2000). Thus, our observation that losartan attenuates the rise in intracellular free Ca$^{2+}$ levels in ischemia may, in part, explain the antiarrhythmic effects of losartan reported in previous studies.

In theory, there are a number of possible mechanisms by which losartan could attenuate the rise in intracellular free Ca$^{2+}$ levels in ischemia. For example, interventions that attenuate acidosis, Na$^+$ loading, or Ca$^{2+}$ influx all may protect against elevations in intracellular Ca$^{2+}$ (Kitakaze et al., 1988; Przyklenk et al., 1987; Tani and Neely, 1989, 1990). Interestingly, angiotensin II has been shown to stimulate Na$^+$-H$^+$ exchange in ischemic hearts and thereby cause intracellular Na$^+$ accumulation (Grace et al., 1996). Intracellular Na$^+$ accumulation leads to intracellular Ca$^{2+}$ accumulation via the Na$^+$-Ca$^{2+}$ exchanger (Tani and Neely, 1989). Thus, it is possible that losartan inhibits Na$^+$-loading in ischemia by blockade of the actions of angiotensin II. However, as our study was conducted in isolated myocytes in the

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**Fig. 10.** Losartan treatment increases Ca$^{2+}$ transients and SR Ca$^{2+}$ stores in the absence of ischemia and reperfusion. A, caffeine-elicited Ca$^{2+}$ transients increased during exposure to losartan and continued to increase following up to 40 min of washout. Caffeine-elicited Ca$^{2+}$ transients were not altered in time-control experiments. Data have been normalized as a percentage of the first response prior to treatment. B, Ca$^{2+}$ transients increased with time in cells exposed to losartan. Data have been normalized as a percentage of the first caffeine-induced Ca$^{2+}$ transient and SR Ca$^{2+}$ transients during 80 min of recording time. C, diastolic Ca$^{2+}$ levels declined gradually in cells that served as time controls but did not decline in cells exposed to losartan. Data have been normalized as a percentage of the first response prior to treatment. *p < 0.05 denotes significantly different from time controls; n = 6 myocytes from six hearts in the time-control group; n = 7 myocytes from five hearts in the losartan group.
absence of exogenous angiotensin II, it would be necessary to postulate that locally produced angiotensin II is responsible for Na\(^+\) and Ca\(^{2+}\) accumulation.

We also demonstrated that losartan abolished the rebound increase in Ca\(^{2+}\) transients in early reperfusion. The mechanism by which losartan inhibits this rebound increase has not yet been established. An increase in the activity of the Na\(^+\)-Ca\(^{2+}\) exchanger may promote Ca\(^{2+}\) entry in early reperfusion. Indeed, studies have shown that oxidative stress and intracellular Ca\(^{2+}\) overload in ischemia increase the activity of the Na\(^+\)-Ca\(^{2+}\) exchange in early reperfusion (Goldhaber and Liu, 1994; Goldhaber, 1996). Losartan decreases oxidative stress (Franco Mdo et al., 2003; Chamorro et al., 2004) and reduces intracellular Ca\(^{2+}\) overload during ischemia. Thus, losartan may decrease the overshoot in Ca\(^{2+}\) transients in early reperfusion by reducing oxidative stress and intracellular Ca\(^{2+}\) overload in ischemia.

We also found that exposure to losartan gradually increased SR Ca\(^{2+}\) stores and increased the magnitude of Ca\(^{2+}\) transients, even in cells that were not exposed to ischemia and reperfusion. These observations suggest that losartan may improve contractile function in reperfusion by increasing SR Ca\(^{2+}\) stores and therefore SR Ca\(^{2+}\) release. Interestingly, signs of Ca\(^{2+}\) overload, such as aftercontractions, afterpotentials, or spontaneous beats, did not accompany this increase in SR Ca\(^{2+}\). The mechanism by which losartan increases SR Ca\(^{2+}\) stores and Ca\(^{2+}\) transients may involve increased SR Ca\(^{2+}\) uptake. Interestingly, Jaiswal et al. (1991) showed that losartan stimulates release of prostacyclin in vascular smooth muscle and neuronal cells by an action independent of angiotensin II receptor blockade. Prostacyclin stimulates SR Ca\(^{2+}\) uptake by increasing phosphorylation of phospholamban, which regulates the SR Ca\(^{2+}\) pump (Doni et al., 1994; Karzewski et al., 1998). Thus, losartan may increase SR Ca\(^{2+}\) stores in cardiac myocytes by stimulation of prostacyclin release, which would increase the activity of the SR Ca\(^{2+}\) ATPase. Alternatively, losartan might increase SR Ca\(^{2+}\) stores by AT\(_1\) receptor blockade.

Angiotensin II binding activates phospholipase C, which releases diacyl glycerol and inositol trisphosphate (Griendling et al., 1987). Inositol trisphosphate releases Ca\(^{2+}\) from the SR in cardiac myocytes (Fukuta et al., 1998). Thus, locally produced angiotensin II could reduce SR Ca\(^{2+}\) stores, and this action would be inhibited by the AT\(_1\) antagonist, losartan. Interestingly, the effects of losartan on SR Ca\(^{2+}\) stores and Ca\(^{2+}\) transients developed slowly and continued even after losartan was removed. This contrasted with the immediate effect of losartan on diastolic free Ca\(^{2+}\) observed during ischemia. The reason for this delay is not clear; however, it is possible that the delay occurs if losartan acts through a signaling pathway in which the levels of intermediates do not return to basal levels quickly. Alternatively, the slowly developing effects may represent accumulation of an active metabolite of losartan, for example, the noncompetitive and potent AT\(_1\) receptor antagonist EXP3174 (Israli, 2000).

To determine whether the protective effects of losartan and its effects on SR Ca\(^{2+}\) load are mediated by AT\(_1\)-receptor blockade or by non-AT\(_1\) receptor mediated actions, one might compare the effects of losartan to other AT\(_1\) receptor antagonists. If the effects of losartan are mediated by AT\(_1\)-receptor blockade, then all AT\(_1\) antagonists would be expected to exert the same effects. However, if the protective effects of losartan represent non-AT\(_1\) receptor actions, only chemically related compounds may exert these effects. For example, the anti-inflammatory and anti-aggregatory effects of losartan, which are not mediated by AT\(_1\)-receptor blockade, are shared by losartan’s metabolite, EXP3174, and by irbesartan, which are chemically similar to losartan (Sadoshima, 2002). In contrast, these actions are absent with candesartan and valsartan, which are chemically dissimilar to losartan (Sadoshima, 2002).

In summary, we found that exposure of field-stimulated cardiac myocytes to losartan during ischemia markedly improved recovery of contractile function in reperfusion. This improvement in contractile function in reperfusion was not attributable to changes in RMP or APD in ischemia and reperfusion. However, losartan prevented the increase in free intracellular Ca\(^{2+}\) levels observed in ischemia, abolished the overshoot in Ca\(^{2+}\) transients in early reperfusion, and increased the magnitude of Ca\(^{2+}\) transients in late reperfusion. This increase in magnitude of Ca\(^{2+}\) transients in late reperfusion was accompanied by an increase in SR Ca\(^{2+}\) stores in losartan-treated myocytes. This increase in Ca\(^{2+}\) transients and SR Ca\(^{2+}\) stores also was observed in cells exposed to losartan in the absence of ischemia and reperfusion. Thus, losartan has important effects on Ca\(^{2+}\) homeostasis under normoxic conditions, and these effects are preserved in cells exposed to ischemia and reperfusion. The beneficial effects of losartan on contractile activity in reperfusion may be related both to prevention of ischemia-induced elevation of diastolic Ca\(^{2+}\) and to increased SR Ca\(^{2+}\) stores. The present study suggests that losartan may exert direct protective effects on the myocardium in patients with ischemic heart disease, in addition to its well-established hemodynamic effects.

Acknowledgments

We thank Peter Nicholl, Cindy Maplebeck, and Steve Foster for excellent technical assistance.

References


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