Losartan Improves Recovery of Contraction and Inhibits Transient Inward Current in a Cellular Model of Cardiac Ischemia and Reperfusion

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ABSTRACT

Losartan, a selective angiotensin II (AII) type I receptor antagonist, may protect against myocardial stunning and arrhythmia in ischemia and reperfusion. To examine the cellular basis for these protective actions, we studied effects of losartan and AII on contractile and electrical activity of ventricular myocytes exposed to simulated ischemia and reperfusion. Ionic currents were measured using voltage-clamp techniques and contractions were measured with a video edge detector. After 10 min of superfusion with Tyrode’s solution at 37°C, cells were exposed to simulated ischemia (hypoxia, acidosis, hyperkalemia, hypercapnia, lactate accumulation, and substrate deprivation) for 30 min followed by 25 min of reperfusion with normal Tyrode’s solution. During ischemia, drug-treated cells were exposed to either 0.1 μM AII, 10 μM losartan, or both simultaneously. In reperfusion, contractions were depressed to 42% of preischemic levels in untreated cells. Losartan treatment significantly improved contractile recovery to 84% (P < .05) of preischemic levels. All-treated cells showed contractile recovery similar to untreated cells (40%), whereas cells treated with losartan plus AII recovered to 101% of preischemic levels. Cells exposed to losartan or losartan plus AII also exhibited reduced incidence of transient inward current (I_{ti}) (20%, P < .05; 36%) relative to untreated cells (60%). However, I_{ti} incidence was not altered by treatment with AII alone (57%). Treatment with exogenous agonist did not potentiate contractile depression or I_{ti} incidence, and losartan exerted protective effects in the presence and absence of AII. Thus, losartan may have effects that are independent of AII receptor blockade.

Evidence that activation of the renin-angiotensin system may exacerbate damage caused by ischemia and reperfusion has been provided by many studies. Angiotensin II (AII) levels are elevated in both heart failure and ischemic heart disease (Sigurdsson et al., 1993; Good et al., 1994), and AII may be synthesized under conditions of ischemia by a cardiac renin-angiotensin system (Tian et al., 1991; So et al., 1998). Furthermore, angiotensin-converting enzyme (ACE) inhibition, which decreases production of AII, has been shown to improve postischemic contractile function in both animal and human studies (Przyklenk and Kloner, 1993, Duncker et al., 1998). ACE inhibition has also been reported to suppress cardiac arrhythmia (Cleland et al., 1985; Webster et al., 1985; Kingma et al., 1986). Thus, inhibition of AII synthesis can be protective in the setting of ischemia and reperfusion.

Several studies have reported that losartan, a selective AII type 1 (AT_1) receptor antagonist, also may exert antiarrhythmic effects and improve contractile function in ischemia and reperfusion (Werrmann and Cohen, 1996; Lee et al., 1997; Matsumoto et al., 1997; Pitt et al., 1997; Paz et al., 1998). However, losartan has also been reported to have deleterious effects on contractile recovery in reperfusion (Ford et al., 1996, 1998). Therefore, it remains unclear whether AT_1 receptor antagonists exert protective effects in ischemic heart disease.

We recently found evidence that losartan may exert antiarrhythmic actions that are independent of AT_1 receptor blockade. We showed that losartan suppressed sustained ventricular tachycardia in an isolated tissue model of ischemia and reperfusion (Thomas et al., 1996). This protective effect was attributed to attenuation of depressed cardiac impulse conduction during ischemia and early reperfusion. Improved transmural conduction likely prevented reentrant arrhythmias in this model. Losartan treatment improved transmural impulse conduction in ischemia both in the presence and absence of exogenous AII. However, AII alone did not promote conduction defects. Thus, Thomas et al. (1996) suggested that losartan may have an intrinsic antiarrhythmic action that is independent of AT_1 receptor blockade.

ABBREVIATIONS: AII, angiotensin II; ACE, angiotensin-converting enzyme; AT_1, receptor, angiotensin II type 1 receptor; I_{ti}, transient inward current; I_{Ca-L}, L-type Ca^{2+} current.
Because intracellular Ca\textsuperscript{2+} is elevated in ischemia and reperfusion, and because elevation of intracellular Ca\textsuperscript{2+} slows impulse conduction (Weingart, 1977; Jalife et al., 1989), we hypothesized that the antiarrhythmic actions of losartan might result from reduced Ca\textsuperscript{2+} overload during ischemia and reperfusion. Signs of intracellular Ca\textsuperscript{2+} overload can be observed in isolated myocytes exposed to simulated ischemia and reperfusion (Cordeiro et al., 1994). In early reperfusion, Ca\textsuperscript{2+} overload frequently induces transient inward current (I_{TI}), a current that is carried by the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and Cl\textsuperscript{-} in ventricular muscle (Kass et al., 1978; Zygmunt et al., 1998). I_{TI} generates oscillatory afterpotentials that can induce triggered arrhythmias (Ferrier, 1977). If losartan decreases Ca\textsuperscript{2+} overload during ischemia and reperfusion, losartan treatment would be expected to decrease incidence of I_{TI} and triggered arrhythmia. Therefore, we investigated whether losartan reduces signs of Ca\textsuperscript{2+} overload in an isolated cardiac myocyte model of ischemia and reperfusion.

Postischemic contractile depression or "stunning" also has been reported previously in animal models of ischemia and reperfusion, and in humans (Duncker et al., 1998). Although mechanisms underlying myocardial stunning are not entirely understood, it is believed that Ca\textsuperscript{2+} overload and oxygen-derived free radicals are key mediators of this phenomenon (Maxwell and Lip, 1997). Because our model of ischemia and reperfusion allows measurement of cell shortening in addition to transmembrane currents (Cordeiro et al., 1994), we investigated whether losartan also might improve contractile function of myocytes in reperfusion.

This study identifies and characterizes effects of losartan in an isolated myocyte model of ischemia and reperfusion that allows examination of direct, nonvascular effects of losartan on myocytes. The specific objectives of this study were as follows: 1) to determine whether losartan affects myocyte contractile function and incidence of I_{TI} in ischemia and reperfusion; and 2) to determine whether protective actions of losartan require the presence of exogenous AII.

**Experimental Procedures**

**Myocyte Isolation.** All experiments were performed in accordance with guidelines published by the Canadian Council on Animal Care. Male guinea pigs (325–375 g; Charles River, St. Constant, Quebec, Canada) were injected with heparin (3.3 IU/g) and anesthetized with sodium pentobarbital (80 mg/kg). After the chest was opened, the heart was rapidly cannulated and perfused retrogradely through the aorta (10–12 ml/min) for 7 to 8 min, with oxygenated (100% O\textsubscript{2}; 36°C) Ca\textsuperscript{2+}-free solution of the following composition: 120 mmol/l NaCl, 3.8 mmol/l KCl, 1.2 mmol/l KH\textsubscript{2}PO\textsubscript{4}, 1.2 mmol/l MgSO\textsubscript{4}, 10 mmol/l HEPES, 11 mmol/l glucose (pH 7.4 with NaOH). Collagenase A (25 mg) and protease (4.8 mg/50 ml; Sigma type XIV) were then included in the perfusate for 5 min. After dissociation, the ventricles were minced and washed in a substrate-enriched, high-K\textsuperscript{+} buffer of the following composition: 80 mmol/l KOH, 50 mmol/l gluta- matic acid, 30 mmol/l KCl, 30 mmol/l KH\textsubscript{2}PO\textsubscript{4}, 20 mmol/l taurine, 10 mmol/l HEPES, 10 mmol/l glucose, 3 mmol/l MgSO\textsubscript{4}, 0.5 mmol/l EGTA (pH 7.4 with KOH). This isolation procedure provided only ventricular myocytes, with no other cell types apparent. Myocytes were plated at a density where most cells were not in contact with others. More than 80% of cells were rod shaped and free of membrane blebs. Myocytes were 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\textsuperscript{-1}, 37°C) with Tyrode’s solution of the following composition: 129 mmol/l NaCl, 20 mmol/l NaHCO\textsubscript{3}, 0.9 mmol/l NaH\textsubscript{2}PO\textsubscript{4}, 4 mmol/l KCl, 0.5 mmol/l MgSO\textsubscript{4}, 2.5 mmol/l CaCl\textsubscript{2}, 5.5 mmol/l glucose, pH 7.4, gassed with 95% O\textsubscript{2}, 5% CO\textsubscript{2}.

**Methods.** Myocytes were visualized with a video camera and TV monitor. Contractions were recorded as unloaded cell shortening with a video edge detector. Discontinuous single-electrode voltage-clamp recordings (sample rate 10–12 kHz) were made with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Recordings were made with high-resistance microelectrodes (18–25 M\textOmega), filled with 2.7 mol/l KCl to minimize dialysis and avoid buffering intracellular Ca\textsuperscript{2+} levels. CLAMP 6.1 software (Axon Instruments) was used to generate voltage-clamp protocols and to acquire and analyze data.

After 10 min of control recordings in Tyrode's solution, cells were superfused for 30 min with an "ischemic" solution designed to mimic conditions associated with ischemia, including hypoxia, hypercapnia, hyperkalemia, acidosis, lactate accumulation, and substrate deprivation (Ferrier et al., 1985, Ferrier and Guyette, 1991). This solution had the following composition: 123 mmol/l NaCl, 6 mmol/l NaHCO\textsubscript{3}, 0.9 mmol/l NaH\textsubscript{2}PO\textsubscript{4}, 8 mmol/l KCl, 0.5 mmol/l MgSO\textsubscript{4}, 2.5 mmol/l CaCl\textsubscript{2}, 20 mmol/l sodium-lactate, gassed with 90% N\textsubscript{2}, 10% CO\textsubscript{2}, pH = 6.8. A 90% N\textsubscript{2}, 10% CO\textsubscript{2} gas phase was layered over the superfusion chamber throughout simulated ischemia. Reperfusion was simulated by return to Tyrode’s solution for 25 min. Each cell was exposed to only one cycle of ischemia and reperfusion, and drugs were added to the superfusate only during ischemia. Drug-treated cells were exposed to either 10 µM losartan, 0.1 µM AII, or losartan plus AII. We have previously shown that losartan blocks effects of AII in the heart at this concentration (Thomas et al., 1996).

**Analyses.** Inward currents were measured with respect to a reference point at the end of the test step. Peak L-type Ca\textsuperscript{2+} current (I_{Ca,L}) was measured as the maximum inward deflection, whereas I_{TI} was measured as the first inward oscillation in current observed during the repolarizing step. Amplitude of contraction was measured as maximum cell shortening with reference to a baseline immediately before the onset of shortening. Contraction-voltage relationships were analyzed with two-way repeated measures ANOVA, whereas time courses were analyzed with a one-way repeated measures ANOVA. Post hoc comparisons were made with a Bonferroni test. Differences in incidence between cell populations were determined with a chi square test. Statistical analyses were performed with Sigma Stat (Jandel, version 1.02). Data are presented as mean ± S.E. The value of n represents the number of myocytes sampled. No more than two myocytes from the same heart were used.

**Compounds.** Losartan was a gift from Merck Frosst Canada Inc. (Kirkland, Quebec, Canada). Collagenase A was obtained from Roche Diagnostics (Laval, Quebec, Canada). All other drugs and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

**Results**

**Effects of Ischemia and Reperfusion on Contractions and Currents.** Figure 1 shows representative recordings of contractions and currents elicited by the voltage-clamp protocol shown at the top. The test step was preceded by a train of ten 200-ms conditioning pulses to 0 mV to provide a history of regular activation. Conditioning pulses were followed by a 500-ms step to −52 mV, to inactivate sodium channels, and a 200-ms test step to −2 mV. Under preischemic conditions (Fig. 1A) myocytes exhibited phasic contractions and I_{Ca,L} in response to the test step. Also shown in Fig. 1, B to D, are recordings made at selected times during the ischemia-reperfusion protocol in the absence of drug. Contractions were almost completely abolished during...
simulated ischemia, but recovered in early reperfusion. In late reperfusion, however, contractions again were depressed. $I_{\text{Ca-L}}$ decreased during ischemia and showed little recovery in late reperfusion.

Effects of Losartan and AII on Postischemic Contractile Depression. Figure 2 shows mean data illustrating the effects of ischemia and reperfusion on contraction amplitude for untreated and drug-treated cells. The voltage-clamp protocol described in Fig. 1 was applied every 5 min throughout the experiment. In untreated cells (Fig. 2A), contractions were markedly reduced during ischemia and then recovered to near preischemic levels in early reperfusion. With continued reperfusion, however, contractions gradually decreased again, and maximal depression occurred at 20 min of reperfusion. All drug-treated groups (Fig. 2, B–D) showed decreases in contraction during ischemia that were similar to untreated cells. However, contractile recovery in reperfusion was affected strongly by drug treatment during ischemia. In fact, reperfusion-induced depression of contraction was abolished by exposure to 10 μM losartan in ischemia (Fig. 2B). Losartan also improved postischemic contractile recovery in the presence of AII (Fig. 2D), even though treatment with 0.1 μM AII alone did not alter the effects of ischemia and reperfusion on contraction (Fig. 2C).

Effects of Losartan and AII on Contraction-Voltage Relationships. We previously have shown that pharmacological agents can alter the voltage dependence of contraction in isolated myocytes (Mason and Ferrier, 1999). Therefore, the increase in amplitude of contractions after losartan treatment in ischemia could result either from increased maximum cell shortening or from a shift in the voltage dependence of contraction. To differentiate between these possibilities, contraction-voltage relationships were determined with the voltage-clamp protocol shown in Fig. 3. The cycle of conditioning pulses plus test step was repeated every 7 s, and with each repetition the test step from $-52$ mV was made more positive in 10-mV increments. Representative recordings of contraction are shown in Fig. 3A for two selected test steps. Under preischemic conditions, contractions showed a sigmoidal relationship with voltage that plateaued near $120$ mV (Fig. 3B). Voltage steps from $-52$ mV were used to inactivate sodium current, but allow activation of various mechanisms believed to contribute to excitation-contraction coupling (Howlett and Ferrier, 1997; Wier and Balke, 1999).

Contraction-voltage relationships were compared in preischemia, ischemia (30 min), and late reperfusion (20 min). Mean contraction-voltage relationships for all treatment groups were normalized to the preischemic contraction amplitudes for each group to compensate for differences between groups before treatment (Figs. 4 and 5). In untreated myocytes, maximum contraction was significantly depressed during ischemia and late reperfusion, without a shift in the
voltage dependence of contraction (Fig. 4A). Losartan-treated cells exhibited reduced contractility during ischemia that was similar to that observed for untreated cells (Fig. 4B). However, in late reperfusion, the mean contraction-voltage relationship was not significantly different from preischemia in either amplitude or voltage dependence. Figure 5A shows that contraction-voltage curves for AII-treated cells were markedly depressed during both ischemia and late reperfusion, and resembled those of untreated cells. However, cells treated with losartan plus AII (Fig. 5B) exhibited decreased contraction amplitude during ischemia, but relatively normal contraction-voltage relationships in late reperfusion. Thus, losartan treatment, in the presence or absence of exogenous AII, increased maximum contraction in late reperfusion but did not shift the voltage dependence of contraction.

**Effects of Losartan and AII on I_{Ca-L}.** The magnitude of I_{Ca-L} was examined for each treatment group during simulated ischemia and reperfusion. The voltage protocol used was similar to that shown in Fig. 1, but with a postconditioning potential of −40 mV to eliminate any contribution from Na\(^+\) current or T-type Ca\(^{2+}\) current. In untreated cells (Fig. 6A), I_{Ca-L} gradually decreased during ischemia, and was significantly reduced from preischemic levels in early reperfusion. A similar time course was observed with losartan (Fig. 6B); however, depression of I_{Ca-L} was not significantly different from preischemic levels. The largest effect on I_{Ca-L} occurred with AII (Fig. 6C). In this group, I_{Ca-L} was significantly reduced at the end of ischemia and throughout reperfusion. However, when cells were exposed to losartan as well as AII, depression of I_{Ca-L} was similar to that of untreated cells and was significantly decreased at only one point in reperfusion (Fig. 6D). Thus, it appeared that losartan attenuated the effect of AII on I_{Ca-L}.

I_{TT}, I_{TT}, was elicited by the voltage protocol shown in Fig. 7A, and was only observed in the first 10 min of reperfusion. Test steps were preceded by 10 conditioning pulses, followed by a 500-ms step to −80 mV. Voltage steps of 300 ms to −40 mV and +20 mV were followed by repolarizing steps to potentials between −100 and +10 mV. Figure 7, B and C, show representative recordings of current and contraction, respectively, for an untreated cell in early reperfusion. I_{TT} appeared as oscillatory downward deflections in current and was accompanied by aftercontractions. The contraction-voltage relationship for aftercontractions (Fig. 7D) reached a peak at −50 mV, and was essentially the mirror image of the current-voltage plot for I_{TT} (Fig. 7E). This current-voltage relation-
ship was very similar to that described in previous studies (Kass et al., 1978; Cordeiro et al., 1992, 1994).

ITI was observed in 60% of untreated cells (Fig. 8). However, in losartan-treated cells the incidence of ITI was significantly reduced to 20%. Although losartan decreased incidence of ITI in the presence of exogenous AII (36%), this effect was not statistically different from preischemic levels. Neither of the AII treatment groups exhibited a shift in the voltage dependence of contraction during ischemia or reperfusion. * denotes \( P < .05 \).

**Discussion**

The purpose of this study was to identify and characterize effects of losartan on the electrical and contractile responses of cardiac myocytes to simulated ischemia and reperfusion. The specific objectives were to determine whether losartan affects contractile function and recovery, and the incidence of ITI in ischemia and reperfusion. In addition, we examined whether these effects require the presence of exogenous AII. We found that losartan treatment improved contractile recovery in reperfusion. The contraction-voltage relationships demonstrated that this protective effect did not result from a shift in the voltage dependence of contraction, but rather from increased maximum amplitude of contraction. Losartan treatment also decreased the incidence of ITI in reperfusion. In contrast, AII did not alter the occurrence of either contractile depression or ITI in reperfusion. Furthermore, the effects of losartan on contraction and ITI were similar in the presence and absence of exogenous AII. AII also increased depression of \( I_{\text{Ca-L}} \) in reperfusion. This effect was attenuated by the addition of 10 \( \mu M \) losartan (D). * denotes \( P < .05 \), \( n = 14 \) to 22 cells/group.
Because I_{\text{rT}} is believed to cause triggered arrhythmias (Ferrier, 1977), the decrease in incidence of I_{\text{rT}} observed with losartan suggests that losartan will suppress triggered arrhythmias in reperfusion. Thus, our results identify a second potential antiarrhythmic action of losartan, in addition to protection against reentry in ischemia and reperfusion, which we reported in our previous study (Thomas et al., 1996). These actions might explain antiarrhythmic effects of losartan reported in studies in animal models of ischemia and reperfusion (Lee et al., 1997; Matsuo et al., 1997) and in humans (Pitt et al., 1997).

Most studies that have reported protective effects of losartan on contractile and electrical activity have attributed these actions to blockade of the renin-angiotensin system. Indeed, several studies have shown that AII can precipitate myocardial injury (Yoshiyama et al., 1994) and arrhythmia (Linz and Scholkens, 1987). In addition, ACE inhibitors improve contractile recovery in reperfusion (Przyklenk and Kloner, 1993; Duncker et al., 1998) and reduce ventricular arrhythmias (Cleland et al., 1985; Webster et al., 1985; Kingma et al., 1986). Taken together, these studies suggest that AII exerts deleterious effects in ischemia and reperfusion that can be attenuated by blocking AII synthesis or actions at AT_{1} receptors.

A role for the AT1 receptor in the actions of losartan is not clear in our study. In the present study we found that AII did not promote I_{\text{rT}} or worsen contractile recovery upon reperfusion. This might be explained if endogenous AII activated Fig. 8. Losartan treatment significantly reduced incidence of I_{\text{rT}}. Cells treated with losartan showed a significant reduction in incidence of I_{\text{rT}} relative to untreated myocytes. The occurrence of I_{\text{rT}} also was reduced in cells treated with losartan plus AII, although this effect was not significant. AII treatment during ischemia had no effect on I_{\text{rT}} incidence (n = 11–20 cells/group).

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AT\textsubscript{1} receptors maximally, and therefore no further effect could occur with addition of exogenous agonist. However, this explanation does not fit with our observations. Addition of exogenous AII reduced I\textsubscript{Ca-L} in reperfusion, and this effect was reversed by losartan. Similarly, in our earlier study, addition of AII significantly affected refractory period in guinea pig ventricle, and this effect also was blocked by 10 \mu M losartan (Thomas et al., 1996). Thus, endogenous AII could not have been exerting maximal effects. Furthermore, losartan had no effects on refractory period in the absence of exogenous AII. Therefore, endogenous AII likely contributed little to the responses of cardiac muscle to ischemia and reperfusion.

In our previous study, 10 \mu M losartan clearly blocked effects of 0.1 \mu M AII on refractory period (Thomas et al., 1996). However, suppression of reentrant arrhythmias by losartan in that model was mediated by changes in transmural conduction, not by changes in refractory period. Losartan affected transmural conduction independently of AII. Therefore, it was concluded that the antiarhythmic effects of losartan might not be mediated by AT\textsubscript{1} receptor blockade. The present study suggests that protective effects of losartan on incidence of I\textsubscript{T} and contractile recovery also likely are independent of AT\textsubscript{1} receptor blockade.

Several studies by others have reported actions of losartan that were not mediated by blockade of the actions of AII (Jaiswal et al., 1991; Bertolino et al., 1994; Chansel et al., 1994). Jaiswal et al. (1991) showed that losartan stimulates release of prostacyclin in vascular smooth muscle and neural cells by an action independent of AII receptor blockade. Prostacyclin has been shown to attenuate myocardial stunning (Farber et al., 1988). In addition, prostacyclin and the prostacyclin analog 7-oxo-PG12 have been reported to have antiarrhythmic effects in ischemia and reperfusion (Fiedler and Mardin, 1986). Whether prostacyclin release contributes to effects of losartan observed in our model remains to be determined.

The present study uses application of losartan in a flow-through system. Therefore, most of the effects of losartan are likely attributable to losartan itself. However, losartan is metabolized to the active metabolite called EXP-3174 (Messerli et al., 1996), which, in theory, could contribute to the protective effects of losartan observed in this study. In future studies, it will be interesting to examine EXP-3174 or other AT\textsubscript{1} receptor antagonists that do not have active metabolites to see whether they have similar protective effects to losartan in ischemia and reperfusion.

Both stunning and I\textsubscript{T} are believed to be promoted by elevated intracellular Ca\textsuperscript{2+} (Matsuda et al., 1982; Duncker et al., 1998). Therefore, the protective effects of losartan observed in our study might reflect attenuation of Ca\textsuperscript{2+} overload during ischemia and reperfusion. Losartan theoretically could reduce Ca\textsuperscript{2+} overload by acting at a variety of sites involved in intracellular Ca\textsuperscript{2+} regulation. Our results suggest that losartan does not affect I\textsubscript{Ca-L}; however, other possible sites of action may include the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger or ATP-dependent Ca\textsuperscript{2+} pumps in the sarcolemma and sarcoplasmic reticulum. Additional experiments are needed to investigate these possibilities.

Because myocytes exposed to simulated ischemia and reperfusion reliably exhibited contractile depression in reperfusion, our model may be useful for investigation of the cellular mechanisms responsible for stunning. An additional advantage of this model is that it allows investigation of the pathophysiology of stunning in the absence of vascular effects that may contribute to dysfunction in whole-heart models (Gao et al., 1995).

The present study provides evidence for several new effects of losartan that might be beneficial in ischemic heart disease. This is the first report of an inhibitory effect of losartan on I\textsubscript{T}, and therefore describes a new mechanism of action by which this drug may suppress reperfusion arrhythmias. This also is the first study to show that losartan can improve postischemic contractile recovery through direct actions on myocytes, and that such actions may be mediated by a mechanism independent of AII. Because both postischemic contractile depression and I\textsubscript{T} are signs of Ca\textsuperscript{2+} overload, we hypothesize that losartan has an action on Ca\textsuperscript{2+} homeostasis in addition to its actions at the AT\textsubscript{1} receptor.

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References


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