Losartan Preserves Integrity of Cardiac Gap Junctions and PGC-1α Gene Expression and Prevents Cellular Apoptosis in Remote Area of Left Ventricular Myocardium Following Acute Myocardial Infarction

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SUMMARY

This study tests the hypothesis that peroxisome proliferator activated receptor-γ coactivator 1α (PGC-1α) and the integrity of gap junctions (GJs) were suppressed and the number of apoptotic bodies was increased in remote viable areas of left ventricle following acute myocardial infarction (AMI), which can be reversed by losartan therapy. Open chest surgery was consecutively performed on 32 adult male Sprague-Dawley rats. These rats were classified into 4 groups (n = 8/each group): group I, AMI (by ligation of left coronary artery (LCA) without treatment); group II, AMI with losartan 20 mg/kg/day; group III, sham control (without LAD ligation); and group IV, sham control with losartan 20 mg/kg/day. Echocardiography was performed on day 1 prior to AMI and on day 14 just before the rats were to be sacrificed for cellular and molecular studies. The results showed that mRNA expression of PGC-1α, integrated area (µm²) of clustered connexin43 (Cx43) spots, and Cx43 GJs were substantially down-regulated and the number of apoptotic bodies was markedly increased in nontreated AMI rats compared with healthy control and losartan-treated AMI rats on day 14 following AMI (all values of P < 0.001). Additionally, day 14 left ventricular (LV) ejection fraction was significantly lower in nontreated AMI rats than in healthy control and losartan-treated AMI rats (all values of P < 0.0001).

Down-regulation of GJs and PGC-1α gene expression and cellular death were frequently observed in remote viable areas of LV following AMI. Losartan therapy reversed the adverse effects of AMI and preserved LV function. (Int Heart J 2007; 48: 533-546)

Key words: Acute myocardial infarction, PGC-1α mRNA expression, Gap junction, Cellular apoptosis, Losartan treatment
Acute myocardial infarction (AMI) is the leading cause of death of patients hospitalized for cardiovascular disease.\textsuperscript{1,2}) Left ventricular (LV) dilatation, and pump failure, following AMI is the principal reason accounting for poor clinical outcomes.\textsuperscript{3–6}) Clinical studies have consistently demonstrated that the severity of pump failure typically depends on infarct size and left ventricular ischemic area\textsuperscript{3–8}) which initiates a cascade of progressive structural and geometric changes in the left ventricle, a process commonly referred to as remodeling. In addition, experimental data have supported the suggestion that damage from AMI is often progressive.\textsuperscript{9})

Basic studies have demonstrated that a variety of pathophysiological stimuli, such as myocardial infarction, hypertension, or dilated cardiomyopathy can increase cardiac workload and elevate mechanical stress on cardiomyocytes.\textsuperscript{10–14}) These findings,\textsuperscript{10–14}) therefore, suggest that living cells are continually challenged by various acute or chronic stresses that in turn, result in cellular apoptosis and death. An animal model study recently demonstrated that the gene expression of peroxisome proliferator activated receptor-\(\gamma\) coactivator 1\(\alpha\) (PGC-1\(\alpha\)) which is involved in the regulation of oxidative metabolism and mitochondrial biogenesis was down-regulated in congestive heart failure (CHF).\textsuperscript{15}) Moreover, gap junctions, which are composed of connexin (Cx) subunits, play a key role in electrical coupling and signal transduction between cardiomyocytes\textsuperscript{16,17}) and changes in these Cx expression patterns have been associated with a variety of cardiac pathologies and contribute to the development of cardiac arrhythmia and programmed cell death.\textsuperscript{18–21}) However, whether AMI affects the integrities of cellular and molecular structure and function of noninfarct areas (defined as remote viable areas) remains unclear. Furthermore, although the pleiotropic effects of losartan have recently been recognized,\textsuperscript{22,23}) little is known regarding the impacts of losartan therapy on the integrity of Cx43 gap junction and mRNA expression of PGC-1\(\alpha\) following AMI.

Therefore, this study tested the hypothesis that PGC-1\(\alpha\), an index of primary regulation of mitochondrial biogenesis and Cx43 gap junction integrity, was significantly suppressed and that the number of apoptotic bodies was notably increased in the remote area of LV following AMI. These, as a consequence, caused LV dysfunction. This study also tested the hypothesis that losartan therapy reversed the adverse effects of AMI and preserved LV function.

Methods

Ethics: All animal experimental procedures were approved by the Animal Care and Use Committee of our hospital and were performed in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23,
Animals, protocol, and procedure: Thirty-two adult male SD rats weighing 250-300 grams (Charles River Technology, BioLASCO Taiwan, Co., Ltd, Taiwan) were utilized and equally divided into 4 groups: group I, AMI without treatment \((n = 8)\); group II, AMI with losartan 20 mg/kg/day, according to a previous experiment\(^{22}\) \((n = 8)\); group III, sham controls \((n = 8)\); and group IV, sham control with losartan 20 mg/kg/day.

Rats were anesthetized by intraperitoneal injections of chloral hydrate (35 mg/kg). Each rat was placed in a supine position on a warming pad at 37°C and then intubated with positive-pressure ventilation (180 mL/min.) with room air using a Small Animal Ventilator (SAR-830/A, CWE, Inc., USA). Under sterile conditions, the heart was exposed through a left thoracotomy for all 4 groups. The proximal left coronary artery was ligated for group I and II rats just below the left atrium with 7-0 prolene sutures. Regional myocardial ischemia was confirmed by observing a rapid change from reddish to a whitish-dark color on the anterior surface of the LV and rapid development of akinesia and dilatation in the at-risk area. Muscle and skin were closed in layers after the procedure. Rats were anesthetized with sodium pentobarbital and sacrificed on day 14 following AMI. The heart was removed and the rats were euthanized by exsanguination. The remote viable areas of LV tissues were harvested, frozen rapidly in liquid nitrogen, and stored at -80°C. Some of the LV myocardium was fixed in 10% buffered formalin for cellular apoptosis study using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) test.

Functional assessment by echocardiography: Transthoracic echocardiography was performed with a commercially available echocardiographic system (UF-750XT) equipped with a 8-MHz linear-array transducer for animals (FUKUDA Denshi Co. Hongo, Bunkyo-ku, Tokyo) while the rats were in the supine position and on day 1 prior to AMI induction and on day 14 just before the rats were to be sacrificed under anesthesia. The heart was imaged in 2-dimensional mode in a short-axis view of the LV at the level of the papillary muscle. This view was used to record M-mode tracings. Left ventricular internal dimensions [end-systolic diameter (ESD) and end-diastolic diameter (EDD)] were measured according to the American Society of Echocardiography leading-edge method using at least 3 consecutives cardiac cycles.\(^{24}\) The LV ejection fraction (LVEF) was calculated as 
\[
\text{LVEF} (\%) = \left(\frac{\text{LVEDD}^3 - \text{LVEDS}^3}{\text{LVEDD}^3}\right) \times 100.
\]
All measurements were performed by an animal cardiologist blinded to the treatment and nontreatment groups.

Immunolabeling of connexin43 and quantitative image data analysis: Six serial sections of LV tissues (3 longitudinal and 3 transverse) were prepared at 4 μm thickness using a Cryostat (Leica CM3050S) for Cx43 immunolabeling. Samples
were fixed in acetone for 15 minutes at -20°C and blocked in a Beta Blocker Kit (Zymed Company, #50-300), solution A and B for 30 minutes and 10 minutes at room temperature, respectively. To colocalize troponin I and Cx43 in the same sample, the tissue sections were first incubated with a mixture of the polyclonal anti-Cx43 (1:200) plus anti-Troponin I (1:200) for 24 hours at 4°C, followed by incubation with anti-mouse FITC (1:200) and anti-rabbit Rhodamine (1:200) for 30 minutes at room temperature, respectively. All experiments with myocardial sections served as positive controls, whereas those without primary antibody served as negative controls.

For each rat, 6 slides were chosen for immunolabeling of Cx43 gap junctions. For each slide, the number of intact Cx43 gap junctions (defined as linear aggregation of Cx43-labeled spots between the cardiomyocytes) was quantified under a fluorescence microscope in 3 randomly chosen HPF. The average number of Cx43-positively stained gap junctions for each animal per high-power field was then obtained by summation of the number of gap junctions and divided by 18.

Calculation of the integrated area (µm²) of Cx43 spots in the slides was achieved using Image Tool 3 (IT3) image analysis software (The University of Texas Health Science Center in San Antonio, UTHSCSA, Image Tool for Window, version 3.0, USA). The quantification procedure for the 3 slides chosen for each rat was described as follows. Three randomly selected HPFs were analyzed in each slide. The number of pixels in each Cx43 spot per HPF was first determined, followed by summation of the pixel numbers obtained from the 3 HPFs in each slide. The procedure was repeated in two other slides for each animal. The mean pixel number per HPF for each animal was then determined by summation of all the pixel numbers divided by 9. The mean area of Cx43 per HPF was obtained by adopting a conversion factor of 19.24 (1 µm² represented 19.24 pixels).

**Western blots for Cx43:** Equal amounts (30 µg) of protein extracts were loaded and separated by SDS-PAGE using 10% acrylamide gradients. After electro-

<table>
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<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>PCR product size (bp)</th>
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<td>PGC-1α</td>
<td>AF049330</td>
<td>AGTGTGCTGCTCTGGTTGGTG</td>
<td>GGAGGGTCATCGTTTGTGGTC</td>
<td>613</td>
</tr>
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*PGC-1α indicates peroxisome proliferator activated receptor-γ coactivator 1α.*
phoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Nonspecific sites were blocked by incubation of the membrane in blocking buffer [5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)] overnight. The membranes were incubated with the indicated primary antibodies (connexin43 1:1000, Zymed), for 1 hour at room temperature. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000, Amersham Biosciences) was used as a second antibody for 1 hour at room temperature. The washing procedure was repeated 8 times within 1 hour, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposure to Biomax L film (Kodak). For purposes of quantitation, ECL signals were digitized using Labwork software (UVP). The same membrane was reprobed with anti-actin antibody after complete removal of antibodies.

**TUNEL assay:** *In situ* DNA fragments were detected using the TUNEL method. Briefly, deparaffinized sections were pretreated with protease K (20 mg/mL) for 15 minutes at room temperature. Sections were incubated with terminal deoxynucleotidyl transferase for 1 hour at 37°C. The reaction was visualized using streptavidin-biotin peroxidase complex (TdT-FragELT M DNA fragmentation detection kit) (Calbiochem) and diaminobenzidine. Normal nuclei with a relatively insignificant number of DNA-3-OH ends cannot be stained using this technique. For negative controls, sections were incubated without the enzyme or nucleotide. For each rat, 6 slides (3 longitudinal and 3 transverse sections) were chosen for TUNEL assays. The average number of TUNEL-positive cells was examined in 3 randomly chosen high-power fields (×400) and normalized to the total number of cells divided by 18.

**Real-time quantitative PCR analysis:** Real-time polymerase chain reaction (PCR) was performed using SYBR Green technology on a Light-Cycler rapid thermal cycler (Roche Diagnostics, Meylan, France). This method, which was used according to the protocol in a previous study, is briefly described as follows.15) Forward and reverse primers (Table I) were each designed in a different exon of the target gene sequence, eliminating the possibility for amplifying genomic DNA. For each set of primers, a basic local alignment search tool (BLAST) indicated that sequence homology was acquired for the target gene only. Optimal condition was conducted for each set of primers, which consisted of determining optimal primer and MgCl₂ concentrations, template concentration, and confirming the efficiency of the amplification. To verify the specificity of the amplification, the PCR product was subjected to a melting curve analysis. Amplification by PCR was performed in duplicate in a total reaction volume of 15 µL. The reaction mixture was comprised of 1 µL diluted template, 1.5 µL of FastStart DNA Master SYBR Green I kit, 3 mM PGC-1α or 4 mM β-actin,
MgCl₂ and 0.5 µM forward and reverse primers. After an 8-minute activation of Taq polymerase, amplification was allowed to proceed for 30-40 cycles, each consisting of denaturation at 95°C for 10 seconds, annealing at 65°C for 5-10 seconds, and extension at 72°C for 5-24 seconds.

**Statistical analysis:** Data are expressed as mean values (± SD). The log transform of an integrated area (µm²) of clustered Cx43 spots and PGC-1α was used to improve the normality. The significance of differences between groups was evaluated by one-way ANOVA followed by Tukey’s multiple comparison procedure. Statistical analysis was performed using SAS statistical software for Windows version 8.2 (SAS Institute, Cary, NC). A probability value < 0.05 was considered statistically significant.

**RESULTS**

Table II shows the body weights and LVEF of 32 rats in two intervals. Initial body weight did not differ among the 4 groups. However, final body weight was
significantly higher in groups III and IV than in groups I and II. Additionally, initial LVEF did not differ among the 4 groups. However, the LVEF was significantly higher in groups III and IV than in groups I and II, and significantly higher
Table III shows mRNA expression of PGC-1α for the 4 groups. The PGC-1α expression (a relative ratio of PGC-1α to β-actin) was substantially lower in group I (0.33 ± 0.15) than groups II (0.84 ± 0.41), III (1.93 ± 0.80) and IV (1.89 ± 0.74). Additionally, the PGC-1α expression was significantly lower in group II than in groups III and IV.

Figure 1 shows the analytical results of quantitative TUNEL detection of apoptotic bodies. The results showed that the number of apoptotic bodies was
remarkably higher in group I than in groups II-IV (all values of $P < 0.001$). Additionally, the number of apoptotic bodies was significantly higher in group II than in groups III and IV ($P < 0.001$). Figure 2 shows the TUNEL-staining method for examining apoptotic bodies. Under a high-power field ($\times 400$), apoptotic bodies were markedly increased in group I compared with those in groups II-IV.

Figure 3 shows the results of quantification of the integrated area ($\mu m^2$) of clustered Cx43 spots in each group of rats. The summation area of Cx43 did not differ among groups II-IV ($P = 0.325$). However, the summation area of Cx43

![Figure 5](image-url)
spots was significantly lower in group I than in groups II-IV ($P < 0.0001$). Figure 4 shows the number of intact Cx43 gap junctions did not differ among groups II-IV ($P = 0.542$). However, the number of intact Cx43 gap junctions was significantly lower in group I than in groups II-IV (all values of $P < 0.001$).

Figure 5 A-D shows the results of the numbers of intact Cx43 gap junctions in each group of rats. As compared with groups II-IV, the number of intact Cx43 gap junctions in group I was notably downregulated following AMI (Figure 5A).

Table IV and Figure 6 show the relative ratio of Cx43 to $\beta$-actin by Western blot examination. The results of Western blot demonstrated that Cx43 protein was significantly lower in group I than in groups II-IV.

**DISCUSSION**

This study has several novel implications. First, the mRNA expression of PGC-1 $\alpha$, integrated area of clustered Cx43 spots, and the number of intact Cx43 gap junctions in the remote LV area and LV function were markedly suppressed following AMI. Second, the number of apoptotic bodies in the remote LV area was notably elevated following AMI. Third, losartan therapy significantly reversed these cellular and molecular perturbations in the remote area and preserved LV function in rats following AMI.

A clinical observational study has demonstrated that AMI slows coronary flow globally, and slower coronary blood flow was associated with regional wall motion abnormalities in distribution of the nonculprit artery. Consistent with this, an experimental study showed that normal myocardium can be damaged in a setting of AMI. Therefore, it is believed that myocardial damage in both ischemic zones and remote areas due to AMI is often progressive. Notably, although the relationship between the infarct size and LV remodeling, LV cham-
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Bar dilatation, CHF and clinical outcomes are well established,\(^3\)\(^-\)\(^8\) the impact of AMI on the integrity of molecular structure and biological activity of the remote LV area has not been further investigated. The principal finding in this study was that AMI markedly down-regulated PGC-1\(\alpha\) activity, which is a major regulator of oxidative metabolism and mitochondrial biogenesis.\(^15\)\(^,\)\(^26\) Additionally, LV function was significantly suppressed following AMI. Therefore, this experimental finding extended the results from another in vitro study\(^9\) and further supports the findings of previous clinical observational studies.\(^3\)\(^-\)\(^8\),\(^25\)

Studies have previously demonstrated that Cx43 gap junctions are clusters of cell membrane aqueous channels linking cytoplasmic compartments of adjacent cells, thereby providing minimal resistance pathways for electrical coupling, direct intercellular exchanges of ions, secondary messengers, and small signaling molecules.\(^16\)\(^-\)\(^20\),\(^27\),\(^28\) Thus, changes in these expression patterns of Cx43 gap junction have been shown to associate with a variety of cardiac pathologies and contribute to the development of cardiac arrhythmia.\(^20\),\(^27\),\(^28\) Furthermore, without cell-to-cell communication, that mediated by gap junctions, could elicit programmed cell death, or “apoptosis” which has been found to increase in a variety of cardiac insults, such as ischemia-reperfusion injury and cardiac failure.\(^29\) Nevertheless, the impact of AMI on connexin43 gap junction expression and cellular apoptosis in remote viable myocardium which undoubtedly plays an essential role for maintenance of heart function following myocardial infarction remains uncertain. Two important findings in the present study were that the integrated area (\(\mu\)m\(^2\)) of clustered Cx43 spots and the number of intact Cx43 gap junctions in the remote LV area were substantially suppressed following AMI. Furthermore, the apoptotic body formation was notably increased in the remote LV area following AMI. Accordingly, our findings reinforced the findings of previous studies\(^16\)\(^-\)\(^20\),\(^27\)\(^-\)\(^29\) and support our hypothesis that AMI elicited molecular and cellular perturbations in remote viable myocardium.

Although the benefits of losartan therapy on improving CHF, LV function, and clinical outcomes have been well recognized in the clinical setting of AMI,\(^30\) the molecular basis of this drug for improving CHF and LV function is currently unclear. Since alternations in mitochondrial biogenesis and energy metabolism following AMI due to insufficient production of PGC-1\(\alpha\) is believed to be a major mechanism leading to decreased cardiac contractility,\(^15\) the finding of this study is of interest in that losartan can reverse the process through an upregulation of PGC-1\(\alpha\) expression. Thus, our finding also extended clinical findings.\(^30\) Another novel finding of this study is that, following AMI, the remote area of LV showed a drastic increase in the number of apoptotic bodies which was substantially suppressed by losartan therapy. Furthermore, the primary strength of the finding in this study was that the adverse effects of AMI on integrated area (\(\mu\)m\(^2\))
of clustered Cx43 spots, the number of intact Cx43 gap junctions, and LV function were abrogated by losartan treatment. These findings, therefore, highlight the impact of losartan therapy on preventing molecular and cellular perturbations and programmed cell death in the LV myocardium following AMI.

It remains uncertain whether such protective effects from losartan are due to the result of an anti-inflammatory effect or other mechanism(s). We propose that the effects of losartan therapy on preventing LV remodeling following AMI, changes in blood pressure and improving systemic circulation may, at least in part, contribute to these molecular cellular protections. Available data from both previous and recent studies showed that hypoxia following myocardial ischemia or mechanical strain was an important role for remodeling of intercellular gap junctions in myocardium. Accordingly, our hypothesis is supported by these previous studies. Interestingly, other studies identified that certain pathophysiological contexts, like hypertrophy, hypoxia, ischemia, pressure overload, and diabetes-related metabolic dysregulation suppressed PGC-1α gene expression. Our finding that AMI decreased PGC-1α gene expression is consistent with the results obtained by these previous studies. Conversely, pioglitazone, a peroxisome proliferator-activated receptor-γ agonist, attenuated LV remodeling and heart failure after experimental MI. Our finding that losartan increased PGC-1α gene expression and preserved LV function is comparable with the results of this study.

The effects of cytokines on immune-mediated myocyte injury and remodeling process of noninfarcted LV myocardium have been studied in depth previously. An experimental study previously demonstrated that in addition to an antihypertensive effect, losartan also has anti-inflammatory properties. Furthermore, another experimental investigation recently showed that the losartan metabolite EXP3179 stimulated endothelial nitric oxide synthetase (eNOS) phosphorylation and suppressed tumor necrotic factorα-induced endothelial cell apoptosis. Perhaps these findings provide some information to support some uncertainty of our study.

This study has one limitation. Utilization of M-mode to assess the global LV function may not be a precise measurement. Therefore, LV function may be overestimated in the present study.

In conclusion, losartan therapy can abrogate the adverse effects of AMI in a remote area of the LV myocardium and preserves LV function.

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