Effect of pravastatin on ventricular arrhythmias in infarcted rats: role of connexin43

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Chen CC, Lien HY, Hsu YJ, Lin CC, Shih CM, Lee TM. Effect of pravastatin on ventricular arrhythmias in infarcted rats: role of connexin43. J Appl Physiol 109: 541–552, 2010. First published May 13, 2010; doi:10.1152/japplphysiol.01070.2009.—Epidemiologic studies showed that men treated with statins appear to have a lower incidence of sudden death than men without statins. However, the specific factor for this remained disappointingly elusive. We assessed whether pravastatin enhanced connexin43 expression after myocardial infarction through attenuation of endothelin-1. Twenty-four hours after ligation of the anterior descending artery, male Wistar rats were randomized to vehicle, pravastatin, mevalonate, bosentan, or a combination of pravastatin and mevalonate or pravastatin and bosentan for 4 wk. Myocardial endothelin-1 levels were significantly elevated in vehicle-treated rats compared with sham-operated rats. Myocardial connexin43 expression at the border zone was significantly decreased in vehicle-treated infarcted rats compared with sham-operated rats. Attenuated connexin43 expression was blunted after administration of pravastatin, as assessed by immunofluorescence analysis, Western blotting, and real-time quantitative RT-PCR of connexin43. Bosentan enhanced connexin43 amount in infarcted rats and did not have additional beneficial effects on pravastatin-treated rats. Arrhythmic scores during programmed stimulation in vehicle-treated rats were significantly higher than scores in those treated with pravastatin. In contrast, the beneficial effects of pravastatin-induced connexin43 were abolished by the addition of mevalonate and a protein kinase C inducer. In addition, the amount of connexin43 showed significant increase after addition of bisindolylmaleimide, implicating that protein kinase C is a relevant target in endothelin-1-mediated connexin43 expression. Thus chronic use of pravastatin after infarction, resulting in enhanced connexin43 amount by attenuation of mevalonate-dependent endothelin-1 through a protein kinase C-dependent pathway, may attenuate the arrhythmogenic response to programmed electrical stimulation.

endothelin-1; myocardial infarction

CARDIAC REMODELING has been associated with myocardial hypertrophy and gap junction heterogeneities (48). Connexin43 (Cx43) is the 43-kDa member of a conserved family of membrane-spanning gap junction proteins, of which Cx43 is the principal junctional protein in mammalian myocardium (5). Gap junction channels provide the basis for the electrical syncytial properties of the heart as a communicating electrical network (15). The gap junction mediates cell-to-cell movement of ions, metabolites, and cell signaling molecules and may play important roles in synchronized vasoactive responses, growth responses, and second-messenger signaling (4). A reduction in gap junctional coupling between myocytes may be an important morphological feature that could induce ventricular arrhythmias in diseased myocardium (4). Decreased ventricular Cx43 levels have been implicated in the pathogenesis of ventricular arrhythmias in humans (16) and knockout mice (7, 13). Recently, engraftment of Cx43-expressing cells has been shown to prevent ventricular arrhythmias after myocardial infarction (MI) (33).

Previous studies have shown a beneficial effect of statins on the recurrence of fatal ventricular arrhythmias in patients with coronary artery disease (26). However, the mechanisms involved in antiarrhythmic effects remain unclear. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the key enzyme that regulates the synthesis of cholesterol from mevalonic acid by suppressing the conversion of HMG-CoA. Mevalonate is the precursor not only of cholesterol but also of many nonsterol compounds; inhibition of HMG-CoA reductase by statins may therefore result in pleiotropic effects (9). Statins may thus exert anti-inflammatory and antiarteriosclerotic actions beyond lipid reduction. We have recently shown (22) that starting statin treatment 24 h after MI substantially reduced ventricular remodeling by attenuation of increased endothelin (ET)-1 expression. ET-1 induces a reduction of Cx43 expression and function in ovarian carcinoma cells and astrocytes (11, 39). However, the role of ET-1 in the regulation of Cx43 in myocardium after MI has not been studied. Therefore, this study aimed 1) to elucidate the possible contribution of pravastatin to cardiac Cx43 modulation, 2) to explore the downstream functional significance of increased Cx43 expression by ventricular pacing, 3) to determine whether ET-1 plays a role in the amount of Cx43 in a rat MI model by the use of bosentan, a nonspecific ET receptor antagonist, and 4) to assess whether protein kinase C (PKC) modulates the amount of ET-1-related Cx43 in a rat MI model by the use of bisindolylmaleimide (BIM), a PKC inhibitor.

METHODOLOGY

The animal experiment was approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals in the Chi-Mei Medical Center.
Animals

Part 1. Male normocholesterolemic Wistar rats that weighed 250–300 g were fed a normal-sodium diet, with a sodium content of 0.32 wt%, and offered tap water ad libitum before the study. On the study day (day 0), 24 h after MI induced by ligation the left anterior descending artery, rats were randomly separated into 6 groups of 10 rats: vehicle; pravastatin (5 mg·kg⁻¹·day⁻¹); mevalonate (50 mg·kg⁻¹·day⁻¹); the nonselective ET receptor antagonist bosentan (mg·kg⁻¹·day⁻¹; Actelion Pharmaceuticals, Allschwil, Switzerland); a combination of pravastatin + mevalonate; or a combination of pravastatin + bosentan. The dose of pravastatin used in this study was derived from previous experiments in which pravastatin restored the infarct size-limiting effect of ischemic preconditioning in hyperlipidemic rabbits (46). The therapeutic efficacy at this dose of bosentan has been previously demonstrated without hypotensive effects (25). The drugs were started 24 h after infarction, because the drugs can exert maximum benefits in this timing window (49). The study duration was designed to be 4 wk because the majority of the myocardial remodeling process in the rat (70–80%) is complete within 3 wk (3). The experimental procedure in the rat heart is surprisingly short, only ~1.3 h (12). The doses of ET-1 (50), BIM (10), pravastatin (42), and PMA (6) have been shown to effectively modulate PKC. At the end of the study, all hearts were used for performing Western blots for Cx43 at the border zone.

Experimental MI

After anesthesia with intraperitoneal ketamine (90 mg/kg), rats were intubated and the anterior descending artery was ligated with a 6-0 silk as in our previous description (20). Sham-operated rats underwent the same procedure except that the suture was passed under the coronary artery and then removed. Mortality in the animals with MI was ~50% within the first 24 h. None of the sham-operated animals died.

Echocardiogram

At 28 days after operation, rats were lightly anesthetized with an intraperitoneal injection of ketamine (25 mg/kg). Echocardiographic measurements were done with a HP Sonos 5500 system with a 15-6L (6–15 MHz, SONOS 5500; Agilent Technologies, Palo Alto, CA) probe as described previously (20). M-mode tracing of the left ventricle (LV) was obtained from the parasternal long-axis view to measure LV end-diastolic diameter dimension and LV end-systolic diameter dimension, and fractional shortening (%) was calculated.

Hemodynamics and Infarct Size Measurements

After echocardiographic study, functional parameters were measured in anesthetized rats at the end of the study. Hemodynamic parameters were measured in rats anesthetized with intraperitoneal ketamine (90 mg/kg) at the end of the study as previously described (20). A polyethylene Millar catheter was inserted into the right carotid artery and connected to a transducer (model SPR-407, Miller Instruments, Houston, TX) to measure LV systolic and diastolic pressure as the mean of measurements of five consecutive pressure cycles. The maximal rate of LV pressure rise (+dP/dt) and decrease (−dP/dt) was measured. After the arterial pressure measurement, the rats were intubated and artificially ventilated with humidified room air supplemented with oxygen for in vivo electrophysiological tests. At completion of the electrophysiological tests, the atria, the right ventricle, and the LV were rinsed in cold physiological saline, weighed, and immediately frozen in liquid nitrogen after coronal sections of the LV were obtained for infarct size estimation. Five-micrometer sections, taken from the equator of the LV, were fixed in 10% formalin and stained with Masson’s trichrome for determination of infarct size. The boundary lengths of the infarcted and noninfarcted endocardial and epicardial surfaces were traced with a planimeter digital image analyzer. Infarct size was calculated as the ratio of the sum of external and internal diameters of LV, as previously described (20). With respect to clinical importance, only rats with large infarction (>50%) were selected for analysis.

Table 1. Cardiac morphology and hemodynamics at end of study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>Vehicle</th>
<th>Pravastatin</th>
<th>Mevalonate</th>
<th>Bosentan</th>
<th>Prav + Meva</th>
<th>Prav + Bos</th>
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<tbody>
<tr>
<td>No. of rats</td>
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<td>12</td>
<td>11</td>
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<td>Body weight, g</td>
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<td>416 ± 18</td>
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<td>395 ± 19</td>
<td>412 ± 18</td>
<td>419 ± 23</td>
<td>415 ± 17</td>
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<tr>
<td>HR, beats/min</td>
<td>413 ± 20</td>
<td>407 ± 16</td>
<td>405 ± 19</td>
<td>418 ± 20</td>
<td>409 ± 21</td>
<td>416 ± 20</td>
<td>402 ± 15</td>
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<tr>
<td>LVESP, mmHg</td>
<td>117 ± 10</td>
<td>108 ± 11</td>
<td>105 ± 12</td>
<td>112 ± 9</td>
<td>106 ± 9</td>
<td>102 ± 8</td>
<td>106 ± 7</td>
</tr>
<tr>
<td>LVEDP, mmHg</td>
<td>5 ± 2</td>
<td>17 ± 2</td>
<td>16 ± 2</td>
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<td>16 ± 3</td>
<td>19 ± 3</td>
<td>17 ± 4*</td>
</tr>
<tr>
<td>+dP/dt, mmHg/s</td>
<td>7,636 ± 462</td>
<td>2,534 ± 382*</td>
<td>4,562 ± 354†</td>
<td>3,052 ± 227*</td>
<td>4,882 ± 454†</td>
<td>2,897 ± 322*</td>
<td>4,983 ± 364†</td>
</tr>
<tr>
<td>−dP/dt, mmHg/s</td>
<td>6,182 ± 372</td>
<td>2,481 ± 423*</td>
<td>3,828 ± 351†</td>
<td>2,783 ± 340*</td>
<td>4,038 ± 365†</td>
<td>2,566 ± 347*</td>
<td>4,285 ± 359†</td>
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<tr>
<td>Infarct size, %</td>
<td>41± 5</td>
<td>40 ± 3</td>
<td>40 ± 4</td>
<td>40 ± 4</td>
<td>42 ± 4</td>
<td>40 ± 4</td>
<td>41 ± 4</td>
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<tr>
<td>LVW/BW, mg/g</td>
<td>2.28 ± 0.15</td>
<td>2.96 ± 0.40*</td>
<td>2.85 ± 0.32*</td>
<td>3.09 ± 0.42*</td>
<td>2.88 ± 0.37*</td>
<td>2.98 ± 0.29*</td>
<td>2.85 ± 0.37*</td>
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<tr>
<td>RVW/BW, mg/g</td>
<td>0.51 ± 0.08</td>
<td>0.67 ± 0.12*</td>
<td>0.57 ± 0.12†</td>
<td>0.73 ± 0.11*</td>
<td>0.54 ± 0.08†</td>
<td>0.69 ± 0.05*</td>
<td>0.58 ± 0.09*</td>
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<tr>
<td>LungW/BW, mg/g</td>
<td>4.23 ± 0.48</td>
<td>5.40 ± 0.55*</td>
<td>4.55 ± 0.49†</td>
<td>5.28 ± 0.36*</td>
<td>4.62 ± 0.53†</td>
<td>5.63 ± 0.35*</td>
<td>4.53 ± 0.46*</td>
</tr>
</tbody>
</table>

Values are means ± SD. BW, body weight; HR, heart rate; LungW, lung weight; LVEDP, left ventricular (LV) end-diastolic pressure; LVESP, LV end-systolic pressure; LVW, LV weight; Meva, mevalonate; Prav, pravastatin; ±dP/dt, rate of LV pressure rise and fall; RVW, right ventricular weight. *P < 0.05 compared with Sham; †P < 0.05 compared with vehicle-, Meva-, and Prav + Meva-treated groups.
In Vivo Electrophysiological Studies

At 28 days after induction of MI, surface electrocardiograms were obtained with 25-gauge subcutaneous electrodes placed in each limb. Heart rate and rhythm were monitored continuously throughout the procedure. Because the residual neural integrity at the infarcted site is one of the determinants of the response to electrical induction of ventricular arrhythmias (14), only rats with the infarcted area of the LV totally replaced by scar tissue were included. Body temperature was maintained at 37°C by a thermostatically controlled heated lamp. Programmed electrical stimulation was performed through electrodes sewn on the epicardial surface of the right ventricular outflow tract. Induced arrhythmias were effected with an electrical Bloom stimulator. The protocol for pacing and an arrhythmia scoring system were used as previously described (22). When multiple forms of arrhythmias occurred in one heart, the highest score was used. The experimental protocols were typically completed within 10 min.

Real-Time RT-PCR of Cx43

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed with samples obtained from the border zone (<2 mm within the infarct) with the TaqMan system (Prism 7700 Sequence Detection System, PE Biosystems) as previously described (22). For Cx43, the primers were 5'-TGAAGGAGGTTGCCAGACA-3' (sense) and 5'-CGTGAGAGTGGGGAAGGACT-3' (antisense). For cyclophilin, the primers were 5'-ATGGTCAACCCACCCGTGTCTCTCG-3' and 5'-CGTGTGAACTCACCACCCCTGACACA-3'. Cyclophilin mRNA was chosen as the internal standard because it is expressed at a relatively constant level in virtually all tissues. Standard curves were plotted with the threshold cycles versus log template quantities. For quantification, Cx43 expression was normalized to the expressed cyclophilin. Reaction conditions were programmed on a computer linked to the detector for 40 cycles of the amplification step. Experiments were replicated three times, and results are expressed as the mean value.

Western Blot Analysis of Cx43

Samples obtained from the border zone were homogenized. Homogenates were centrifuged at 10,000 g for 30 min to pellet the particulate fractions. The supernatant protein concentration was determined with a bicinchoninic acid (BCA) protein assay reagent kit (Pierce Endogen, Rockford, IL). Twenty micrograms of protein was determined with a bicinchoninic acid (BCA) protein assay reagent kit (Pierce Endogen, Rockford, IL). Twenty micrograms of protein was separated by 10% SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The nitrocellulose membrane was then rinsed with PBS and mounted in Dako fluorescent mounting medium. Isotype-identically different conjugated antibodies served as a negative control.

To investigate the spatial distribution and quantification of Cx43, analysis of immunofluorescent staining was performed on LV muscle from the border zone. Paraffin-embedded sections were produced at a thickness of 5 μm. Tissues were incubated with Cx43 antibodies (1:200; 71-0700, Zymed) in 0.5% BSA in PBS overnight at 37°C. The second antibody was monoclonal goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC, Sigma), at 1:50 dilution in PBS containing 0.5% BSA for 1 h. The sections were washed three times with PBS and mounted in Dako fluorescent mounting medium. Isotype-identically different conjugated antibodies served as a negative control.

The density of Cx43-labeled regions was qualitatively estimated from 10 randomly selected fields at a magnification of ×400. The density was measured on the tracings by computerized planimetry (Image Pro Plus, Media Cybernetics, Silver Spring, MD) as described previously (21). The value was expressed as the ratio of Cx43-labeled area to total area. The slides were coded so that the investigator was blinded to the rat identification.

Table 2. Plasma cholesterol and ET-1 concentrations at end of study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>Vehicle</th>
<th>Pravastatin</th>
<th>Mevalonate</th>
<th>Bosentan</th>
<th>Prav + Meva</th>
<th>Prav + Bos</th>
<th>Border LV ET-1, pg/mg tissue</th>
<th>Remote LV ET-1, pg/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>11</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Plasma cholesterol, mg/dl</td>
<td>47 ± 15</td>
<td>42 ± 13</td>
<td>44 ± 11</td>
<td>42 ± 5</td>
<td>40 ± 8</td>
<td>43 ± 7</td>
<td>40 ± 10</td>
<td>2.0 ± 0.3</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>Plasma ET-1, pg/ml</td>
<td>0.5 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>0.4 ± 0.3</td>
<td>0.9 ± 0.4</td>
<td>0.8 ± 0.4</td>
<td>0.8 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.7</td>
<td>0.2 ± 0.7</td>
</tr>
<tr>
<td>Border LV ET-1, pg/mg tissue</td>
<td>1.5 ± 0.7</td>
<td>4.8 ± 2.1*</td>
<td>3.2 ± 2.0†</td>
<td>5.2 ± 2.5*</td>
<td>4.5 ± 1.5*</td>
<td>5.0 ± 1.6*</td>
<td>3.0 ± 1.8†</td>
<td>2.6 ± 1.5‡</td>
<td>1.5 ± 0.7‡</td>
</tr>
<tr>
<td>Remote LV ET-1, pg/mg tissue</td>
<td></td>
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</table>

Values are means ± SD. ET-1, endothelin-1. *P < 0.05 compared with Sham; †P < 0.05 compared with vehicle-, Meva-, and Prav + Meva-treated groups; ‡P < 0.05 compared with samples from border zone within same group.

Fig. 1. Representative Masson trichrome-stained section of a vehicle-treated heart at 4 wk after infarction (blue color, from 12 to 7 o’clock).
Morphometric Histological Determination of Myocyte Size

The effect of pravastatin on ventricular remodeling after infarction was further confirmed by pathological examination. To exclude differences in cardiomyocyte size in different regions of the LV (38), we obtained samples from the remote region (>2 mm outside the infarct) and used hematoxylin and eosin stain. For consistency of results, myocytes positioned perpendicularly to the plane of the section with a visible nucleus and a clearly outlined and unbroken cell membrane were selected for the cross-sectional area measurement, as previously described (24). This area was determined by manual tracing of the cell contour on a digitized image acquired on the image analysis system at ×400 magnification with computerized planimetry (Image Pro Plus, Media Cybernetics), as described previously (24). A total of 100 myocytes were selected in the LV of each heart and analyzed by an observer blinded to the experimental treatment.

Protein Kinase C Activity Assay

Myocardial muscles from the border zone were mechanically homogenized in PKC extraction buffer (25 mmol/l Tris·HCl, pH 7.4, 0.5 mmol/l ethylenediaminetetraacetic acid, 0.5 mmol/l ethylene glycol tetraacetic acid, 0.05% Triton X-100, 10 mmol/l β-mercaptoethanol, 1 μg/ml leupeptin and aprotinin) and centrifuged at 5,000 g. The activity of purified PKC from the supernatant obtained from vehicle-

Fig. 2. A–G: morphometric analyses of left ventricular (LV) sections from the remote zone at 4 wk after infarction (magnification ×400). LV cardiomyocyte cross-sectional areas in rats treated with indicated agents were examined by hematoxylin and eosin staining. Relative myocyte cross-sectional area was normalized to a mean value of sham-operated rats at the end of the study. A: sham. B: vehicle. C: pravastatin (Prav). D: mevalonate (Meva). E: bosentan (Bos). F: Prav + Meva. G: Prav + Bos. Bar, 50 μm. H: quantitative analysis of cardiomyocyte size in different treated groups. The number of animals in each group is indicated in parentheses. *P < 0.05 compared with vehicle-, Meva-, and Prav + Meva-treated groups; †P < 0.05 compared with infarcted groups.
and pravastatin-treated infarcted rats was measured with a colorimetric PKC activity assay kit (Stressgen Bioreagents, Victoria, BC, Canada) per the manufacturer’s instructions. Briefly, a readily PKC phosphorylated substrate (cyclic adenosine monophosphate response element binding protein) was precoated on the wells of a PKC substrate microtiter plate provided in the kit. The purified PKC samples were added to the wells, and a PKC phosphorylation reaction was initiated with addition of adenosine triphosphate. After a 90-min incubation at 30°C, the reaction was terminated by emptying the contents of each well. A phosphospecific substrate antibody was added to each well, followed by a peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody. After incubation, tetramethylbenzidine substrate was added to develop the reaction. The intensity of the color was measured on a microplate reader at 450 nm, and the relative kinase activity (compared with vehicle) of the samples was calculated from the absorbance measurements.

**Plasma and Tissue Levels of ET-1 and Plasma Cholesterol Levels**

Because of a local release of ET-1 at the border zone, blood samples from the aortic root and the tissue from the border zone and remote zone were obtained for measurements of systemic and local ET-1 levels at the end of the study. Plasma ET-1 concentration was measured by collecting 4 ml of blood in test tubes containing 2% ethylenediaminetetraacetic acid (80 μl/ml of blood). Blood samples were immediately centrifuged at 3,000 g for 10 min, and the plasmas were stored at −70°C until further analysis. ET-1 was measured by immunobead assay (R&D Systems, Minneapolis, MN). Cholesterol was measured in plasma by an automated method.

**Statistical Analysis**

Results are presented as means ± SD. Statistical analysis was performed with the SPSS statistical package (version 11.0, SPSS,
Chicag o, IL). Differences among the groups of rats were tested by one-way ANOVA. Subsequent analysis for significant differences between two groups was performed with a multiple comparison test (Scheffé’s method). Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal-Wallis test followed by a Mann-Whitney test. The significance level was set at the value of $P < 0.05$.

RESULTS

No differences in mortality between vehicle and treated groups were found throughout the study. No sham-operated rats had evidence of cardiac damage. Blood pressure, heart rate, and infarct size did not differ among the infarcted groups (Table 1).

Pravastatin did not lower serum cholesterol in rats (Table 2), consistent with the notion that compensatory increases in hepatic enzyme production were observed in rats treated with statins.

Morphometric Studies

Four weeks after MI, the infarcted area of the LV was very thin and was totally replaced by fully differentiated scar tissue (Fig. 1). The vehicle-, mevalonate-, and pravastatin + mevalonate-treated groups had an increase in right ventricular weight-to-body weight ratio and lung weight-to-body weight ratio compared with pravastatin-, bosentan, and pravastatin + bosentan-treated groups. The weight of the LV inclusive of the septum remained essentially constant 4 wk after coronary artery occlusion among the infarcted groups.

To further confirm cardiac hypertrophy after infarction, cardiomyocyte sizes were measured by histological cross-sectional areas. The cardiomyocytes from the border zone in the vehicle group significantly increased by 65% compared with those from the same area of sham-operated hearts ($P < 0.0001$). Pravastatin reduced cell areas 30% compared with the vehicle group ($P < 0.0001$). Conversely, the rats in which mevalonate was administered developed greater cardiomyocyte hypertrophy than that in the pravastatin-alone-treated group. Treatment with bosentan attenuated ventricular hypertrophy in infarcted rats by 37% compared with the vehicle group, a figure similar to that in the pravastatin-treated group (Fig. 2). However, addition of bosentan did not further attenuate ventricular hypertrophy in pravastatin-treated rats.

Echocardiographic Data

After 4 wk of MI, LV structure and function were evaluated in vivo by echocardiographic analysis. Compared with sham-operated hearts, MI hearts showed structural changes such as increased LV diastolic and systolic diameters (Fig. 3 and Table 3), consistent with LV remodeling. Both LV end-diastolic dimension and LV end-systolic dimension in rats with MI were significantly reduced by pravastatin, bosentan, or pravastatin + bosentan treatments ($P < 0.0001$). LV fractional shortening was significantly higher in the pravastatin-, bosentan-, and pravastatin + bosentan-treated groups compared with the vehicle group. Conversely, the rats in which mevalonate was administered developed more impaired LV systolic function and progressive LV dilation than that in the pravastatin-treated group. These data were corroborated by the results that $+dP/dt$ and $-dP/dt$ were significantly improved in the pravastatin-treated group compared with the pravastatin + mevalonate group.

Circulating and Myocardial ET-1 Levels and PKC Activity

Circulating ET-1 levels remained similar among the groups (Table 2). To investigate the possible role of cardiac ET-1 synthesis, we determined the ventricular ET-1 levels. LV ET-1 levels were significantly upregulated 3.2-fold at the border zone in vehicle-treated rats compared with sham-operated rats (4.8 ± 2.1 vs. 1.5 ± 0.7 pg/mg tissue, $P < 0.0001$). Expression was region dependent, with a significant increase at the border zone (4.8 ± 2.1 pg/mg tissue) compared with that in the interventricular septum (2.6 ± 1.5 pg/mg tissue, $P < 0.0001$) in the vehicle group, consistent with cardiac remodeling beginning from adjacent to remote noninfarcted myocardium. Compared with vehicle-treated rats, in pravastatin-treated rats LV ET-1 levels were significantly lower both at the border and at remote zones. The beneficial effect of pravastatin on LV ET-1 levels was abolished by administration of mevalonate.

Compared with vehicle-treated infarcted rats, rats treated with pravastatin significantly decreased PKC activity after MI (56 ± 5% of vehicle, $P < 0.05$).

Immunofluorescent Studies of Cx43

In the sham-operated group, sections stained with the Cx43 antibody produced intense punctate labeling primarily at intercalated disks between cardiomyocytes, consistent with the gap junctions. Infarction resulted in a significant decrease in total amount of Cx43-immunoreactive signals of 65%. These signals at intercalated disks were significantly higher in animals treated with pravastatin. The ratio of Cx43 area to total area was significantly increased by 63% in pravastatin-treated rats compared with vehicle-treated rats (Fig. 4). The increased expression of pravastatin-related Cx43 could be reversed to normal levels in pravastatin + bosentan- and pravastatin + mevalonate-treated groups.

Table 3. Echocardiographic findings

<table>
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<th>Parameters</th>
<th>Sham Vehicle</th>
<th>Vehicle</th>
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<th>Mevalonate</th>
<th>Bosentan</th>
<th>Prav + Meva</th>
<th>Prav + Bos</th>
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<tr>
<td>Interventricular septum, mm</td>
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<td>7.3 ± 0.2* +</td>
<td>8.7 ± 0.2*</td>
<td>7.4 ± 0.2* +</td>
<td>8.8 ± 0.2*</td>
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<td>LVESD, mm</td>
<td>3.7 ± 0.2</td>
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<td>5.1 ± 0.3* +</td>
<td>7.4 ± 0.2*</td>
<td>5.4 ± 0.3* +</td>
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<tr>
<td>LV posterior wall, mm</td>
<td>1.6 ± 0.2</td>
<td>1.8 ± 0.2*</td>
<td>1.7 ± 0.1</td>
<td>2.0 ± 0.2*</td>
<td>1.8 ± 0.2*</td>
<td>2.2 ± 0.2*</td>
<td>1.9 ± 0.2*</td>
</tr>
<tr>
<td>FS, %</td>
<td>37 ± 2</td>
<td>17 ± 4*</td>
<td>26 ± 3* +</td>
<td>18 ± 3*</td>
<td>30 ± 3*</td>
<td>16 ± 4*</td>
<td>28 ± 3* +</td>
</tr>
</tbody>
</table>

Values are means ± SD. FS, fractional shortening; LVEDD, LV end-diastolic dimension; LVESD, LV end-systolic dimension; other abbreviations as in Table 1. *$P < 0.05$ compared with Sham group; †$P < 0.05$ compared with Infarcted groups treated with vehicle, Meva, and Prav + Meva.
levels similar to those in vehicle-treated rats after addition of mevalonate.

**Western Blot and Real-Time PCR of Cx43**

Two predominant forms of Cx43 were detected (Fig. 5): a nonphosphorylated form (P0) and a phosphorylated species (P1). The P1-to-P0 ratio was used as the magnitude of the phosphorylation of Cx43. Western blot analysis derived from the border zone revealed that P1/P0 was significantly decreased in response to infarction. Ventricular remodeling is associated with progressively decreased amount of phosphorylated and total Cx43 in the border zone. In the pravastatin-treated group, the level of the total amount of Cx43 was maintained at 76% of that in the sham-operated group, 47% higher than that in the vehicle-treated group. Furthermore, P1/P0 was significantly increased in the pravastatin-treated group compared with the vehicle-treated group (8.02 ± 4.02 vs. 1.51 ± 2.08, P < 0.001). Western blot data were consistent with immunohistochemical data analysis.

To elucidate the role of ET-1-related PKC in modulating Cx43 expression, BIM was assessed in an in vitro model. Figure 6 shows that BIM significantly enhanced expression of Cx43 and increased P1/P0 compared with the vehicle group, confirming the role of PKC.
in mediating Cx43 expression. In contrast, PMA abolished the attenuated reduction of pravastatin-induced Cx43 (Fig. 7), implying the role of PKC in modulating Cx43 expression.

To determine whether the preserved Cx43 in the border zone of pravastatin-treated rats was due to an upregulation at the mRNA level, Cx43 mRNA levels were quantified by real-time PCR. Cx43 mRNA levels showed a significant downregulation in the border zone in vehicle compared with sham-operated rats (Fig. 8). In either pravastatin- or bosentan-treated infarcted rats, the Cx43 mRNA levels were significantly increased over those in the vehicle-treated rats. The increased magnitude of Cx43 mRNA levels was blunted by the combination therapy of pravastatin/H11001 mevalonate compared with treatment with pravastatin alone. Cx43 protein expression was in parallel with the mRNA level changes, implying that Cx43 synthesis is a critical regulation step for gap junction protein.

Electrophysiological Stimulation

To further elucidate the physiological effect of enhanced Cx43 expression, ventricular pacing was performed. Arrhythmia score in sham-operated rats was very low (0; Fig. 9).
contrast, ventricular tachyarrhythmias consisting of ventricular tachycardia and ventricular fibrillation were inducible by programmed stimulation in infarcted rats. Pravastatin significantly decreased the inducibility of ventricular tachyarrhythmias compared with vehicle. The beneficial effect of pravastatin on arrhythmic score was abolished by administration of mevalonate.

**DISCUSSION**

Our present study shows for the first time that chronic treatment for 4 wk with pravastatin leads to attenuation of Cx43 amount reduction, probably through a mevalonate-dependent ET-1-PKC pathway in infarcted rats. These results were concordant with beneficial effects of pravastatin, as documented structurally by increase in immunofluorescence-stained Cx43, molecularly by myocardial Cx43 protein and mRNA levels, biochemically by tissue ET-1 levels and PKC activity, and electrophysiologically by improvement of fatal ventricular tachyarrhythmias.

The beneficial effect of pravastatin on arrhythmias was supported by three lines of evidence (Fig. 10): 1) Pravastatin attenuated ET-1 levels. The present results confirmed our previous finding (22) that ET-1 level was significantly increased after infarction. The addition of mevalonate to pravastatin-treated rats impaired not only their ability to attenuate cardiomyocyte hypertrophy but also their ability to suppress ET-1 levels. Thus blocking the mevalonate pathway is a critical step in the mechanism of pravastatin-induced attenuation of ET-1 levels. Mevalonate not only is involved in the synthesis of cholesterol but also is the precursor for the synthesis of isoprenoids. Isoprenoids are essential for the function of signal transduction molecules of the Rho family (18). Regulation of Rho activity by statins is separate from that of statins on lipids. Inhibition of Rho signaling by statins can activate peroxisome proliferator-activated receptors (PPARs; Ref. 8). Activation of PPARγ is beneficial in preventing cardiac ET-1 secretion by inhibiting the AP-1 signaling pathway (8). 2) ET-1 inhibition attenuated reduced Cx43 expression through a PKC-dependent pathway in chronically infarcted hearts. BIM has been shown to be specific for inhibition of PKC isoforms α, β, δ, and γ (43). ET-1-induced attenuated Cx43 expression can be reversed by BIM, strengthening the hypothesis that ET-1-induced inhibition of Cx43 involves PKC. The observation was further supported by the finding that a PKC inducer, PMA, abolished the attenuated reduction of pravastatin-induced Cx43. Indeed, our results were consistent with the findings of Sirnes et al. (37), showing that PKC activation by 12-O-tetradecanoylphorbol-13-acetate attenuated Cx43 expression. Previous studies have shown that ligand-dependent activation of ETA receptor results in activation of a pertussis toxin-insensitive G protein that stimulates phospholipase C activity and activates PKC (35). Furthermore, bosentan administration did not further increase Cx43 expression in pravastatin-treated rats, suggesting a common pathway between both agents. Pravastatin enhanced Cx43 expression through a PKC-dependent pathway. In support of this idea, PKC ELISA data demonstrated that pravastatin attenuated PKC activity and the beneficial effect of pravastatin on Cx43 expression can be abolished by adding PMA, a PKC inducer. 3) The severity of pacing-induced fatal arrhythmias was associated with the amount of Cx43. Our
results showed that diminished Cx43 protein expression may contribute to initiation of fatal arrhythmias in the presence of a healed infarct. Previous studies have shown that attenuated Cx43 expression in the border zone was associated with an increase in wave break and alteration of reentrant wave front (28). Cardiac-restricted Cx43 conditional knockout mice significantly increased incidence of lethal tachyarrhythmias (7). Furthermore, an increased Cx43 amount caused by either heavy ion radiation (2) or cytokines (17) improves the conductivity, decreases the spatial heterogeneity of repolarization, and reduces the vulnerability of infarcted hearts to fatal arrhythmias. Therefore, although the precise mechanism by which pravastatin modulates the amount of Cx43 remains unknown, it is most likely that pravastatin exerts its antiarrhythmogenic effects on myocardium through the preserved amount of Cx43.

Mechanisms

The effect of ET-1 on the changes in Cx43 amount remained sparse and revealed conflicting results (11, 27, 32, 39). Polontchouk et al. (32) have shown that ET-1 administration increases Cx43 in terms of the expression assessed by Western blot and the function assessed by junctional conductance in neonatal rat cardiomyocytes. In contrast, other investigators showed different results that ET-1 reduced Cx43 amount in tumors expressing gap junction (44) and in endothelial cells (45). Thus it seems important to take tissue specificity into account. Furthermore, the opposite results between our study and that of Polontchouk et al. (32) in cells and intact animals suggest that the complex effects of ET-1 on cardiac Cx43 are probably affected by other neurohormonal factors.

Administration of pravastatin not only attenuated reduced Cx43 expression but also prevented ventricular remodeling. The hypertrophic growth of cardiomyocytes may create a variety of changes in Cx43 expression. The overall amount of Cx43 protein was reported to be reduced in human patients with severe aortic stenosis (29) but elevated in the early phase of hypertrophy in guinea pigs with renovascular hypertension. On the other hand, in rats with pressure-overloaded hearts induced by monocrotaline treatment or by aortic banding the cellular content of Cx43 was found to be unchanged compared with that in control hearts (47). One possible explanation of such contradictory results may include the use of different species, sampling periods, and models in which hypertrophy may develop at different rates. No previous studies have shown the relation between Cx43 expression and post-MI-induced cardiomyocyte hypertrophy. Further work is required to differentiate whether there is an effect of pravastatin on the gap junction or on the attenuated cardiomyocyte hypertrophy directly with a secondary change in gap junction.

Other Mechanisms

Although the present study suggests that the mechanisms of pravastatin-induced antiarrhythmia are related to increased Cx43 expression, other potential mechanisms need to be studied. Previous studies have suggested that statins inhibit fatal arrhythmias by directly inhibiting electrophysiological alterations (23, 36), and by preventing cardiac fibrosis (19), thereby reducing the risk of isolated regional slowing of conduction and reentrant arrhythmias. We (23) and others (36) have shown that statins modulated ATP-sensitive K⁺ channels and Ca²⁺-activated K⁺ channels, both of which are important for the induction of abnormal automaticity and reentrant arrhythmias. Prevention of ionic remodeling may be an upstream approach to antiarrhythmic therapy.

Clinical Implications

Pravastatin administration attenuated reduced Cx43 levels after infarction. Our results suggested that pravastatin increases the expression of Cx43 protein and mRNA. The beneficial effects of pravastatin were abolished by addition of mevalonate, implying the important role of HMG-CoA reductase in regulating Cx43 expression. Indeed, our results were consistent with previous findings showing that statins upregulated Cx43 amount in tumors expressing gap junction (44) and in endothelial cells (45).

Although caution should be taken in extrapolating to humans results obtained with experimental animals, the particular pattern of beneficial structural effects is unique for statins and may have an important clinical impact. If, as has been suggested, the alterations in gap junction expression are central to myocardial arrhythmogenicity in the diseased state, a therapeutic strategy may be to correct the underexpression of the gap junctional connexins by the administration of statins. Early treatment with statins should be a routine part of the care of
patients admitted to hospital at the acute phase of MI for providing favorable ventricular remodeling and reducing arrhythmic severity. Besides, pravastatin has been used clinically and has few side effects (30). Existing antiarrhythmic therapy is hampered by a lack of efficacy and unacceptable side effects. Ventricular tachycardia and fibrillation remains the strongest predictor of in-hospital mortality in patients with MI. Because of the relatively few side effects of pravastatin and its protective function, it may be an agent that could be administered to patients after MI to limit detrimental effects of ventricular arrhythmias. The antiarrhythmic effect of pravastatin may be of greater therapeutic benefit in preventing fatal ventricular arrhythmias in patients with MI than traditional antiarrhythmic drugs, which are limited by low efficiency, proarrhythmic effects, or impairment of the quality of life (41).

Study Limitations

There are some limitations in the present study that have to be acknowledged. First, only rats with large infarcts (>30%) were examined, raising the possibility that the reaction of the surviving myocytes may be different with smaller infarcts. Sakai et al. (34) have shown that there was a good correlation between the extent of infarct size and ET-1 levels. Because activation of the ET-1 system is a prerequisite for the pravastatin effect, our finding cannot necessarily be extrapolated to animals with small to moderate infarction. Second, in addition to Cx43, Cx45 and Cx40 are also present in the heart, but their expression was not measured. Spatially defined patterns of expression of three connexin isoforms form the cell-to-cell conduction pathways responsible for the orderly spread of current flow that governs the normal cardiac rhythm. However, Cx45 and Cx40 are much less abundant than Cx43 in working ventricular myocytes. Thus their role in ventricular arrhythmias after infarction has not been defined and needs further study. Third, although BIM has been used extensively as a “specific” inhibitor of PKC, BIM also inhibited the activity of the RSK2 isoform of the 90-kDa ribosomal S6 kinase (p90RSK) family (1). Nonspecific inhibition of p90RSK isoform(s) at PKC-inhibitory concentrations would complicate the interpretation of data obtained with BIM. This would be of particular concern when investigating the roles of PKC isoforms in (patho)physiological processes, such as Cx43 expression, where p90RSK activity is also likely to play a role (31). Nevertheless, the relative effects of BIM on the activities of p90RSK versus PKC in cardiac myocytes are unknown. Fourth, although we demonstrated that pravastatin attenuated the reduction of Cx43 amount probably through an ET-1-dependent pathway in infarcted rats, we did not provide direct evidence by evaluating the effect of pravastatin on Cx43 expression via ET-1 reconstitution or ETA receptor agonism. Finally, the animal study may not directly represent the patient’s situation. The drug effects of permanent coronary occlusion in the rat model and late patency of the infarct-related artery in most clinical settings on Cx43 expression may be different.

Conclusions

These data show that pravastatin, via a mevalonate-dependent ET-1 pathway, plays an important role in Cx43 expression after infarction. These effects are functionally and structurally important because they are linked to attenuated incidence of fatal arrhythmias. Pravastatin may provide a new strategy for the prevention of postinfarction ventricular arrhythmias.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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