Chapter 7

Improvement of EDHF by Chronic ACE Inhibition Declines Rapidly after Withdrawal in Rats with Myocardial Infarction.

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Abstract

Introduction: Heart failure after myocardial infarction (MI) is associated with endothelial dysfunction. There is conflicting evidence on the exact nature of this endothelial dysfunction, and how endothelium-dependent vasodilation is affected by angiotensin-converting enzyme inhibitor (ACE-I) therapy. Furthermore, consequences of acute ACE-I withdrawal are largely unknown. Therefore, we studied the contribution of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) to the effects of ACE-I therapy and its withdrawal on endothelial function in MI-rats.

Methods: Rats were subjected to coronary ligation to induce MI, and were assigned to quinapril or vehicle from 2 weeks to 8 months post-MI. In parallel, MI-rats treated for 14 months with quinapril were subjected to treatment withdrawal for 0, 4, and 6 weeks. Acetylcholine (ACh)-induced relaxation and underlying endothelium-derived mediators were studied in isolated aortic rings.

Results: Long-term quinapril (8 months) resulted in markedly improved endothelium-dependent vasodilation in rats with myocardial infarction, which could be attributed to marked improvement in non-NO/prostanoids-mediated relaxation (i.e. EDHF). After 14 months of follow-up, maximum vasodilation was still preserved by quinapril. Withdrawal after 14 months treatment caused significantly impaired ACh-induced EDHF-mediated relaxation within 4 weeks. A marked reduction in EDHF mediated relaxation caused this impairment. NO-mediated relaxation was unaffected.

Discussion: These findings highlight the importance of EDHF impairment in development of endothelial dysfunction after myocardial infarction, and the possibility to improve EDHF-mediated vasodilation with chronic ACE inhibitor therapy. In addition, withdrawal of chronic ACE inhibition after MI should be considered carefully, as profound endothelial dysfunction may develop rapidly.
**Introduction**

The progression of left ventricular (LV) dysfunction to chronic heart failure after myocardial infarction (MI) is associated with increased peripheral vascular resistance, which is thought to be the combined result of neurohormonal activation as well as endothelial dysfunction\(^1,2\) and alterations in myogenic tone of resistance arteries\(^3\). However, there is conflicting evidence on the exact nature of endothelial dysfunction in chronic heart failure after MI\(^4-9\). Endothelium-dependent relaxation is mainly mediated by nitric oxide (NO), vasoactive prostaglandins, and endothelium-derived hyperpolarizing factor (EDHF). Alterations of these individual components may be explained by e.g. severity of LV dysfunction, and differences between vessel beds.

Angiotensin-converting enzyme inhibitors (ACE-I) effectively inhibit the progression of LV dysfunction towards overt heart failure. Importantly, ACE inhibition beneficially affects the blood vessels - predominantly by improvement of endothelial function\(^10\). However it has not been fully elucidated which mediators of endothelium-dependent vasodilation are improved by ACE-I therapy. Conversely, little is known about the consequences of acute therapy withdrawal hereon. Interestingly in this context, the antihypertensive effects of ACE inhibition may be sustained for a prolonged period of time after withdrawal of therapy\(^11,12\). However, in spontaneously hypertensive rats, ACE-I withdrawal dissociated between sustained blood pressure lowering after 4 weeks versus loss of improvement of endothelial function at the same moment\(^13\). Hence, in a setting of chronic ACE inhibition after MI, such a rapid loss of the vasoprotective effects of ACE-I after withdrawal may impose a considerable early risk of adverse events. Previous experimental findings in rats with MI\(^14\) as well as clinical observations\(^15,16\) showed the occurrence of endothelial dysfunction and ischemia-related events within weeks after cessation of therapy. The aim of the current study was to examine the effects of long-term treatment with the ACE-I quinapril, and its subsequent withdrawal on different mediators of endothelium-dependent relaxation in rats with myocardial infarction.

**Methods**

*Study design*

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (Published by the US National Institutes of Health, NIH publication No. 85-23, revised 1996), and the animal research committee of the University of Groningen approved this study protocol.

Male Sprague Dawley rats (Harlan, Zeist, The Netherlands), weighing 280±25g were subjected to coronary ligation to induce MI, or sham surgery, as previously described\(^17\). Mortality within the first 24 hours after surgery was 51% for MI rats and 0% for sham rats. Two weeks after coronary ligation, rats were randomized to quinapril (15 mg kg\(^{-1}\) day\(^{-1}\), mixed through food), or vehicle. After 8 months of treatment part of the rats were sacrificed. In the remaining rats treatment was continued up to 14 months.
For the withdrawal study, surviving rats after 14 months of therapy were randomized into 3 groups, which were sacrificed at: 1) end of therapy; 2) 14 months of therapy + 4 weeks withdrawal; 3) 14 months of therapy + 6 weeks withdrawal. This study is an extension of a previous survival study on optimization of ACE-I therapy in rats with MI, and the rationale for the duration treatment for 8 and 14 months was driven by considerations provided in that publication. Moreover, we intended to sacrifice groups of rats at regular time intervals (i.e. 4 and 8 weeks) after withdrawal. However, as we observed clear manifestations of heart failure and a trend of increased mortality within the first weeks, and to anticipate that high mortality would lead to a very limited group size, we decided to sacrifice rats already at 6 weeks after withdrawal instead of 8 weeks.

**Blood pressure**
At the time of sacrifice, rats were anaesthetized as described above, the carotid artery was cannulated and a pressure tip catheter (Micro-Tip 3French, Millar instruments Inc., Houston TX, USA) connected to a 486 PC equipped with an AD converter and appropriate software (Millar instruments, Germany) was advanced into the aortic arch, and arterial blood pressure was recorded.

**Plasma N-ANP**
After blood pressure measurements, arterial blood was collected, anti-coagulated with EDTA, and centrifuged at 4000 RPM for 10 minutes at 4 °C. Plasma was stored at -80 °C until assay. Samples were transported on dry ice to the Core Laboratory at the University Hospital Dijkzigt, Rotterdam, The Netherlands, where all measurements were performed as previously. Concentrations of N-terminal atrial natriuretic peptide (N-ANP) in plasma were measured with a commercially available radioimmunoassay from Biotop (Oulu, Finland).

**Tissue processing/histology**
Hearts were excised and rinsed with ice cold NaCl (0.3%). The atria and right ventricle were removed on ice for determination of left ventricular weights. A midventricular slice of the LV was stored in 2% paraformaldehyde for histological measurements. Infarct size was determined by histology according to methods described before. In brief, the sections were embedded in paraffin, and 5 µm slices were cut and stained with Sirius red/Fast green. Size of the infarct was determined by planimetry, and was expressed as the percentage of scar to total LV circumference. The midventricular section provides adequate estimation of total left ventricular infarct size. Only infarcted rats with MI sizes larger than 20% of LV were included in analysis, since smaller infarcts are reported to be hemodynamically fully compensated in this model; accordingly rats from the present study showing MI<20 % could not be distinguished from sham rats.

**Aorta experiments**
After excision of the heart, the thoracic aorta was removed, immediately cleaned of adhering tissue, and cut into rings of 2 mm in length. Subsequently, rings were mounted on a setup for measurements of isotonic displacements at 1.4 g preload. The
aorta sections were situated in an organ bath containing Krebs buffer: (in mmol L\(^{-1}\)) NaCl (120.4), KCl (5.9), CaCl\(_2\) (2.5), MgCl\(_2\) (1.2), glucose (11.5), and NaHCO\(_3\) (25.0). The organ baths were continuously gassed with a mixture of 95% O\(_2\) and 5% CO\(_2\), and kept at a temperature of 37.5\(^\circ\)C. After a stabilization period of at least 1 h, rings were primed with 60 mM KCl followed by repeated washing and renewed stabilization. Subsequently, rings were precontracted with 1 \(\mu\)mol L\(^{-1}\) phenylephrine (PE), and relaxation responses to increasing concentrations of acetylcholine (ACh, 10 nmol L\(^{-1}\) to 0.1 mmol L\(^{-1}\)) were determined. Apart from total response to ACh, relaxation was measured in presence of 0.1 mM \(\text{N}^{\text{G}}\)-mono-methyl-L-arginine (L-NMMA) to determine NO-mediated vasorelaxation. In pilot experiments at our lab, it was established that this concentration was sufficient to prevent NO-mediated relaxation, as increasing dose or adding another NO-inhibitor (0.1 mM L-NAME) did not further inhibit ACh relaxation. Relaxation in presence of L-NMMA was measured with and without addition of 10\(\mu\)mol/L indomethacin to verify contribution of vasoactive prostanoids. The remaining ACh-evoked relaxation in the presence of L-NMMA and indomethacin was considered an estimate of EDHF contribution to total ACh relaxation, in accordance with previous studies demonstrating its complete abrogation in the additional presence of charybdotoxin and apamin\(^{9,23,24}\). Incubations with L-NMMA and indomethacin were started 30 minutes prior to precontractions with PE; neither of these two drugs induced vasoconstriction or –dilation within these 30 minutes. After the dose–response curves to ACh, the maximum response to the NO donor sodium nitrite (SN, 10 mmol L\(^{-1}\)) was determined.

**Drugs**
Krebs buffers and drug solutions were freshly prepared daily. All compounds were purchased from Sigma (St. Louis, MO, USA).

**Statistics**
Data are presented as means±SEM in case of normal distribution, otherwise as median and range. In case of normal distribution, groups were compared using one-way analysis of variances (ANOVA) with least squared differences post hoc analysis for multiple comparisons in SPSS 10.0. In case of non-normal distribution, a Kruskall–Wallis test with subsequent Mann–Whitney tests for individual group comparison was performed. Dose–response curves were compared with ANOVA for repeated measurements. Differences were considered significant at the level of 0.05 (two tailed). The area under the curve for each concentration–response curve was calculated using SigmaPlot 8.0, and expressed in arbitrary units to express the total response to ACh, and response in presence of L-NMMA, to express the NO- and EDHF-mediated relaxations (figure 2).
Results

General characteristics
The groups of MI-rats sacrificed after 8 months of treatment with quinapril or vehicle, respectively, had comparable infarct sizes (table 1). Groups of MI-rats treated for 14 months with quinapril and sacrificed at 0, 4, and 6 weeks after withdrawal, respectively, were also balanced for infarct size, although the latter three groups all tended to have larger infarct sizes than the 8 month-groups.

Systolic blood pressure of MI-rats was significantly reduced after 8 months of quinapril treatment as compared to those untreated, and was similar after 8 and 14 months of quinapril treatment. Mean systolic blood pressures had increased back to baseline after 4 weeks of withdrawal, and did not change between 4 and 6 weeks.

Untreated MI-rats showed increased left ventricle:body weight (LV:BW) ratios and elevated plasma N-ANP concentrations, demonstrating development of left ventricular hypertrophy and heart failure. Quinapril treatment restored these parameters after 8 months of therapy (table 1). LV:BW ratios were comparable after 8 and 14 months quinapril, but increased significantly after withdrawal. N-ANP concentrations were higher after 14 than 8 months of quinapril, and tended to increase further after withdrawal.

After 8 months, a trend towards increased plasma aldosterone levels in rats with MI compared to sham rats was observed. Eight months quinapril treatment significantly reduced aldosterone levels. After 14 months quinapril still effectively reduced aldosterone levels, as indicated by the marked and significant increase after both 4 and 6 weeks of withdrawal.

Table 1. General characteristics, and cardiac function of sacrificed rats with myocardial infarction after 8 and 14 months chronic quinapril therapy, and the effects of 4 and 6 weeks withdrawal.

<table>
<thead>
<tr>
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<th>8 months</th>
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<th>14 months</th>
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<tr>
<td></td>
<td>Sham</td>
<td>MI</td>
<td>MI + QUI</td>
<td>MI + QUI</td>
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<tr>
<td>N</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Infarct size (%LV)</td>
<td>0±0</td>
<td>27±3</td>
<td>25±1</td>
<td>31±4</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>133±12</td>
<td>125±9</td>
<td>92±2 *</td>
<td>100±7</td>
</tr>
<tr>
<td>LV-BW (mg/g)</td>
<td>2.1±0.1</td>
<td>2.4±0.1 *</td>
<td>2.0±0.1</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Plasma N-ANP</td>
<td>0.8±0.1</td>
<td>1.9±0.2 *</td>
<td>0.8±0.1</td>
<td>2.6±0.4</td>
</tr>
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$dP/dt$ values are shown in $(10^3 \text{mm Hg/sec})/\text{mmHg},$ and were corrected for systolic pressures. N-ANP; N-terminal Atrial Natriuretic Peptide, pmol/mL. * $p<0.05$ versus 8 months sham, † $p<0.05$ versus 14 months QUI.
Total endothelium-dependent vasodilation to ACh
Eight months after onset of treatment, the concentration-response curve for endothelium-dependent relaxation to ACh was significantly decreased in untreated MI-rats as compared to untreated age-matched sham-controls (left panel figure 1A); when calculated as the AUC the decrease in total relaxation to ACh amounted 40% (figure 2A). Quinapril significantly improved total relaxation to ACh; not only when compared to untreated MI-rats, but also when compared to untreated sham control rats (figures 1A and 2A).

The improved response to ACh after 8 months of quinapril was also maintained after 14 months of treatment (right panel figure 1A). However, subsequent cessation of ACE-I treatment significantly impaired total relaxation to ACh, with similar reductions at 4 and 6 weeks after withdrawal (figures 1A and 2A). The worsened response to ACh after ACE-I withdrawal could not be restored by acute incubation of aorta rings with an ACE-I (10 µM quinapril) or an AT₁-receptor antagonist (1 µM candesartan, data not shown).

Endothelial mediators in ACh-induced relaxation
A similar pattern of group differences in concentration-response curves at 8 months after onset of treatment was seen when responses were studied in the combined presence of L-NMMA and indomethacin to block production of NO and vasoactive prostanoid, respectively. These findings indicate a reduced contribution of a non-NO non-prostanoid factor to ACh-induced relaxation in untreated MI-rats, interpreted as reduced EDHF (left panel figure 1B); when calculated as the AUC the decreased EDHF contribution in total relaxation to ACh amounted approximately 30%, compared to untreated age-matched sham-controls (figure 2A). In contrast, the L-NMMA-sensitive contribution in total relaxation to ACh was similar in all groups, indicating that NO-contribution was neither affected by MI, nor by quinapril treatment (figure 2A). Furthermore, the indomethacin-sensitive contribution was negligible in all groups, indicating that vasoactive prostanoids played no role major role in ACh-induced relaxation in the aorta.

Treatment of MI-rats with quinapril for 8 months significantly improved the contribution of EDHF in total ACh-induced relaxation, both when compared to untreated MI-rats as well as untreated sham rats, and this improvement appeared still preserved after 14 months of quinapril treatment. In accordance with that, subsequent cessation of ACE-I therapy caused a rapid and marked deterioration in EDHF, with similar reductions at 4 and 6 weeks after withdrawal (figure 1B). Note that the contribution of EDHF to total ACh-induced relaxation after ACE-I withdrawal in quinapril treated MI-rats was as low as in untreated MI-rats at 8 months after infarction (figure 2A).

Endothelium-independent vasodilation
Maximum endothelium-independent relaxation to the exogenous NO donor sodium nitrite (SN) at 8 months follow-up was similar in untreated sham- and MI-rats (figure 3). Quinapril significantly increased the response to SN at 8 months, and this effect was
Figure 1. Maximum acetylcholine-induced vasodilation after 8 months MI, effects of long-term quinapril treatment, and of 4 and 6 weeks of therapy withdrawal in aorta segments of rats with MI. A) Total relaxation, B) Relaxation in presence of L-NMMA and indomethacin to block NO- and prostaglandin-mediated vasorelaxation. Responses were calculated as percentage of precontraction with $10^{-6}$ mol L$^{-1}$ phenylephrine. L-NMMA: L-methyl arginin ester. *: p<0.05 vs. sham. †: p<0.05 versus 14 months quinapril.
maintained at 14 months. Four weeks after withdrawal, maximum SN-mediated vasodilation had significantly decreased. Between 4 and 6 weeks of withdrawal, maximum vasodilation to SN further decreased significantly. Note that such alterations in vascular vasodilator responsiveness may partially account for the observed reduced response to ACh in MI-rats without (or withdrawn from) quinapril treatment. Nevertheless, correcting the ACh response for the maximum response to SN in an attempt to take this into account did not alter the pattern: the contribution of EDHF in ACh-induced relaxation was still significantly impaired in untreated MI-rats and those in which ACE-I had been withdrawn (see inset figure 2B). Responses to ACh were corrected for differences in response to SN by considering the response to SN as 100% relaxation, and subsequently recalculating ACh-induced vasodilation as a percentage of the difference between PE and SN response.

Discussion

The current study evaluates effects of chronic ACE-I therapy and its withdrawal on endothelial function in rats with myocardial infarction. Main findings of this study are that long-term ACE inhibition with quinapril improved non-NO non-prostanoid...
mediated (i.e. EDHF-mediated) endothelium-dependent vasorelaxation and that within 4 weeks of withdrawal this beneficial effect completely disappeared.

**General characteristics**

Blood pressure was considerably decreased by quinapril, and after 4 weeks of withdrawal, this effect had reversed. The antihypertensive effect of chronic ACE inhibition is generally sustained for weeks after withdrawal, although some studies in hypertensive rats also show lack of sustained blood pressure reduction.\(^{25,26}\) Hence it has been suggested that ACE-I dose and duration of therapy may determine whether or not a rebound blood pressure increment occurs shortly after withdrawal, where the higher dosages and longer treatment periods may be associated with more pronounced blood pressure increases.\(^{26,27}\) Moreover, in the present study, as the rats were supposed to be initially normotensive or hypotensive due to MI, and the rather quick reversal of blood pressure reduction after withdrawal may as well be related to the disease model; MI versus hypertension.

![Figure 3. Vasodilation in response to the nitric oxide donor sodium nitrite (SN, 10 mmol/L). Response is depicted as percentage of relaxation after a precontraction with 1 µmol/L phenylephrine (PE). *: p<0.05 versus sham, †: p<0.05 versus 14 months QUI, ‡: p<0.05 versus 4 weeks withdrawal.](image)

**Endothelium-dependent vasodilation**

Combined data from literature suggest that endothelial function is progressively impaired during LV dysfunction post-MI, and ACE-I therapy results in improved endothelial function. However, the effects of ACE inhibition on the contribution of the different mediators of endothelium-dependent vasodilation are not undisputed. In the present study the ACE-inhibitor quinapril restored the contribution of non-L-NMMA/indomethacin-mediated endothelium-dependent vasorelaxation. This remaining component of relaxation, insensitive to L-NMMA (NO) and indomethacin (prostanoids), is likely to be EDHF-mediated. Although the exact nature of EDHF is not completely elucidated, and may be potassium ions\(^ {28}\), electric signaling via gap junctions\(^ {28}\), or epoxyeicosatrienoic acids\(^ {29}\), all EDHFs exert their vasodilating effect through opening of Ca\(^ {2+}\)-dependent K\(^ +\) (K\(_{Ca}\)) channels. Importantly, previous studies
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demonstrated complete abrogation of the L-NMMA plus indomethacin-resistant vasodilation in the presence of the K_{Ca}-blockers charybdotoxin and apamin\(^9,23,24\). Involvement herein of a NO pool generally insensitive to eNOS blockade cannot be fully excluded, although little is known about such NO-pools. However, involvement of a specific non-L-NMMA-sensitive pool of NO seems very unlikely, as experiments from a previous study showed that adding another NO-inhibitor (0.1 mM L-NAME) did not further inhibit ACh relaxation\(^9\).

Previous studies showed that EDHF contributes substantially to endothelium-dependent relaxation, not only in large conductance arteries, but also in coronary arteries and small resistance arteries\(^31-33\). As alterations in the latter vessels may play a central role in increasing peripheral vascular resistance and hence development and progression of LV dysfunction, EDHF is an interesting potential therapeutic target. However, the involvement of EDHF in endothelial dysfunction during chronic heart failure has gained little attention yet and is still controversial. One study reports increased EDHF compensating for reduced NO-mediated vasorelaxation in isolated mesenteric arteries during experimental heart failure\(^5\). The discrepancy with our present results may be explained by use of different vessels and particularly the much shorter follow-up time of 4-8 weeks after MI induction in that study, as endothelial dysfunction develops over longer periods of time in the rat MI model\(^34\). Indeed, our results are in general accordance with a study with a longer follow-up in the same experimental model showing that aortic endothelial dysfunction was due to impaired EDHF\(^9\).

The exact mechanism underlying EDHF improvement by quinapril and subsequent deterioration after withdrawal cannot be determined from the present study. Blood pressure reduction by ACE inhibition may play a role, but EDHF was also impaired in untreated MI rats compared to sham. As blood pressures were similar between these latter two groups, alterations in endothelial function independent of blood pressure are more likely to underlie the observed effects of quinapril. Rather, local vascular RAAS effects may be involved. Our findings are in line with previous findings showing that RAAS blockade with either ACE inhibition or angiotensin II receptor antagonists improved EDHF in age-related endothelial dysfunction\(^35-37\).

The link between RAAS (blockade) and EDHF is unknown. However, angiotensin II inhibits opening of K_{Ca} channels through AT\(_1\) receptor activation\(^38,39\). Thus RAAS inhibition would augment vasodilation mediated via K_{Ca} channels. Furthermore RAAS inhibition could have decreased sub-endothelial thickening, thereby improving transfer of EDHF to the vascular smooth muscle cells in the media\(^36\).

Withdrawal of long-term quinapril treatment resulted in endothelial dysfunction within 4 weeks. This aortic endothelial dysfunction observed after cessation of quinapril was also explained by loss of EDHF-mediated vasodilation. Again, increased RAAS activity after withdrawal of ACE inhibition\(^14,40\) is likely to underlie the deterioration in EDHF-mediated vasodilation.
**Endothelium-independent vasodilation**

Responsiveness of the aorta to the NO donor sodium nitrite (SN) was increased by quinapril, whereas myocardial infarction itself had no significant effect. The underlying mechanism cannot be determined from the present study did not directly assess responsiveness of the vascular smooth muscle in absence of endothelium. Possibly, ACE inhibition improved cGMP signaling in vascular smooth muscle cells, by increased soluble guanylyl cyclase expression. Part of the improvement in ACh-induced vasodilation by ACE inhibition could thus be explained by effects on the smooth muscle level. However, correcting the ACh response for the maximum response to SN in an attempt to take this into account did not alter the pattern: the contribution of EDHF in ACh-induced relaxation was still significantly impaired in untreated MI-rats and those in which ACE-I had been withdrawn. These findings suggest that changes in smooth muscle responsiveness to vasodilators are unlikely to fully account for the improvement of EDHF-mediated responses by quinapril.

**Study limitations**

The groups analyzed at 8 months had on average smaller infarctions than the groups analyzed at 14 months and subjected to 0, 4, and 6 weeks withdrawal, and this hampers direct comparison in time. The above differences in MI-size may be attributed to infarct expansion due to chronic increased filling pressures as a consequence of progressive LV dysfunction.

The three withdrawal-groups (0, 4, 6 weeks) were harvested at different time points after coronary ligation. This could be a confounding factor when comparing different time points in the course of heart failure development. However, the order of magnitude of difference in time points of sacrifice (weeks) is limited compared to the total treatment period (>1 year).

**Conclusion**

Long-term ACE inhibition in rats with myocardial infarction markedly improved non-NO/prostanoid-mediated (i.e. EDHF) endothelium-dependent vasorelaxation. Within four weeks after treatment withdrawal this beneficial effect completely disappeared. These findings highlight the potential importance of EDHF impairment in development of endothelial dysfunction after myocardial infarction, and the possibility to improve EDHF-mediated vasodilation with chronic ACE inhibitor therapy. In addition, withdrawal of ACE inhibition in patients with LV dysfunction after MI should be considered carefully, as profound endothelial dysfunction develops rapidly and could account for adverse effects of ACE-I withdrawal seen in patients with post-MI LV dysfunction. The mechanism by which quinapril may enhance EDHF requires further study.

**References**


