Monocyte Chemoattractant Proteins Mediate Myocardial Microvascular Dysfunction in Swine Renovascular Hypertension

Jing Lin, Xiangyang Zhu, Alejandro R. Chade, Kyra L. Jordan, Ronit Lavi, Elena Daghini, Matthew E. Gibson, Angelo Guglielmotti, Amir Lerman, Lilach O. Lerman

Background—Monocyte chemoattractant proteins (MCPs) play an important role in mediating inflammatory processes. Hypertension (HTN) is associated with inflammation as well as impaired cardiac microcirculatory function and structure, but the contribution of MCPs to these alterations remained unclear. This study tested the hypothesis that MCPs regulate cardiac microvascular function and structure in experimental HTN.

Methods and Results—Pigs (n=6 per group) were studied after 10 weeks of normal, renovascular HTN, or renovascular HTN+ bindarit (MCPs inhibitor, 50 mg/kg/d PO). Left ventricular (LV) function, myocardial microvascular permeability, and fractional vascular volume were assessed by fast computed tomography before and after adenosine infusion (400 μg/kg/min). Myocardial fibrosis, inflammation, and microvascular remodeling were determined ex vivo. Hypertension was not altered by bindarit, but LV hypertrophy and diastolic function were improved. In response to adenosine, myocardial microvascular permeability increased in HTN (from 0.0083±0.0009 to 0.0103±0.0011 AU, P=0.038 versus baseline) and fractional vascular volume decreased, whereas both remained unchanged in normal and HTN+bindarit pigs. HTN upregulated endothelin-1 expression, myocardial inflammation, and microvascular wall thickening, which were inhibited by bindarit.

Conclusions—MCPs partly mediate myocardial inflammation, fibrosis, vascular remodeling, and impaired vascular integrity induced by hypertension. Inhibition of MCPs could potentially be a therapeutic target in hypertensive cardiomyopathy. (Arterioscler Thromb Vasc Biol. 2009;29:1810-1816.)

Key Words: MCPs | inflammation | hypertension | microvascular permeability | remodeling

Hypertension (HTN) is a leading cause of congestive heart failure in the United States and impairs left ventricular (LV) function, myocardial perfusion, and microvascular function.1,2 Myocardial microvascular dysfunction is an important modulator of coronary resistance and myocardial blood flow3 and is often associated with changes in microvascular architecture like rarefaction or thickening.4 Important attributes of microvascular function include microvascular permeability (MP) and fractional vascular volume (FVV), key indices of microvascular structural integrity. MP, the rate of leakage of plasma components to the extravascular tissue, reflects endothelial barrier function.5,6 FVV, the volume of myocardium occupied by microvessels, represents the number and tone of functional microvessels and myocardial perfusion.7,8 MP and FVV are difficult to assess in vivo but can be accurately and noninvasively assessed by fast CT.2,7

The mechanism by which HTN leads to cardiomyopathy may involve inflammation. Perivascular inflammation of intramyocardial arteries is an early response to pressure overload, including induction of monocyte chemoattractant proteins (MCPs) and macrophage infiltration, in particular MCP-1.9,10 The expression of MCP-1 and its receptor (CCR2) in HTN is regulated by mechanical strain and by release of reactive oxygen species. Furthermore, MCPs mediate vascular inflammation and remodeling by facilitating the secretion of vasoconstrictors like endothelin (ET)-1,11 cytokines, and chemokines, and may contribute to vascular endothelial dysfunction.12,13 However, the role of MCPs in HTN-induced alterations of myocardial microvascular structure and function are poorly understood. The current study was designed to test the hypothesis that MCPs contribute to the impairment of the myocardial microcirculation, and their blockade would improve myocardial microvascular structure and function in swine renovascular HTN.
Methods

Animal procedures were approved by the Institutional Animal Care and Use Committee. Female domestic pigs (initially weighing 25 to 35 kg) (Park Partners, Stewartaeville, Minn.) were randomized into 3 groups: normal (n=6), renovascular HTN (n=6), and HTN pigs supplemented with bindarit (Angelini Research Center - ACRAF, Italy, 50 mg/kg/d P.O., n=6). Bindarit (2-Methyl-2-[1-(phenylimethyl)-1H-indazol-3yl]methoxy)[propanoic acid) is a specific inhibitor of MCP 1, 2, and 3 synthesis,14,15 and at this dose inhibits MCP synthesis and inflammation.16 Renovascular HTN was elicited by induction of renal artery stenosis, which increases arterial pressure within 7 to 10 days.17,18 Mean arterial pressure (MAP) was measured by a PhysioTel telemetry system (Data Sciences) implanted at baseline in the left femoral artery. After 10 weeks, pigs were anesthetized (ketamine 15.7 mg/kg and xylazine 2.3 mg/kg/h in saline), intubated, and ventilated. A pigtail catheter in the right atrium served for contrast media injection and a side-arm for adenosine infusion. Blood samples were collected for measurement of plasma renin activity (PRA), aldosterone, and ET-1 by enzyme immunoassay.19 Fast CT studies were then performed to assess cardiac function and structure in vivo, MP and FVV (before and after adenosine), LV filling rate, and LV muscle mass (LVM). A few days later the pigs were euthanized using pento-barbital (100 mg/kg), and hearts were harvested for in vitro studies. LV myocardial segments were fresh-frozen or preserved in formalin, and another segment prepared for micro-CT studies. Microvascular architecture was assessed by evaluation of microvascular density and wall thickness, inflammation by the expression of MCP-1, its receptor CCR2, cyclooxygenase (COX)-1, COX-2, IL-6, and macrophage infiltration, and cardiac remodeling by myocardial expression of the angiotensin II receptor type I (AT1R), ET-1, interstitial fibrosis, and myocyte hypertrophy. Vascular integrity was also evaluated by Rho-kinase (ROCK) activity (tested by its downstream product phospho-myosin phosphatase) and tight junction protein expression. Because HTN and inflammation can increase oxidative stress, dihydroethidium (DHE) staining for superoxide production and the expression of the p47 and p67 subunits of NAD(P)H oxidase were assessed.20 To evaluate direct effects of MCP-1, cell culture studies were performed using human cardiac fibroblasts (HCF) incubated with MCP-1 (for details, see the supplemental materials, available online at http://atvb.ahajournals.org).

In Vivo CT Studies

Pigs were scanned by either electron beam (C-150, Imatron) or 64-slice multidetector (Somatom Sensation-64, Siemens) fast CT, which provide very similar assessments of MP and FVV,7 and images analyzed with ANALYZE (Biomedical Imaging Resource, Mayo Clinic; see supplemental materials).

In Vitro Studies

Micro-CT was used to evaluate myocardial microvascular density.21,22 Myocardial remodeling was evaluated by myocyte cross-sectional areas, and fibrosis with H&E and trichrome staining. Microvascular remodeling was assessed by microvascular wall thickness to lumen ratio using antihuman α-smooth muscle actin (SMA) (DakoCytona- tion) staining. Immunohistochemistry assessed indices of inflammation and MP, with primary antibodies against MCP-1, macrophage CD163, zonula occludens-1 (ZO-1), and phospho-myosin phosphatase targeting subunit (Thr506pMYP1). The tight junction protein ZO-1 regulates endothelial barrier function and overexpresses in response to strain,23 whereas phospho-MYP1 reflects ROCK activity, which also regulates endothelial barrier function by inactivation of Myosin Phosphatase.24 Western blotting was used for detecting MCP-1, CCR2, IL-6, COX1, COX-2, AT1R, ET-1, endothelial nitric oxide synthase (eNOS), p47 and p67, and ZO-1. GAPDH was used as loading control. For further details, please see the supplemental materials.

Statistical Analysis

Results are presented as mean±SEM. One-way ANOVA with the Bonferroni correction evaluated differences among the groups fol-

### Table. Systemic and Cardiac Function and Myocardial Microvascular Density (mean±SEM) Assessed by Micro-CT in Normal, Hypertension (HTN), and HTN+Bindarit Pigs

<table>
<thead>
<tr>
<th></th>
<th>Normal n=6</th>
<th>HTN n=6</th>
<th>HTN+Bindarit n=6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>101±4</td>
<td>120±5*</td>
<td>124±9*</td>
</tr>
<tr>
<td>Change postadenosine, %</td>
<td>-6±1‡</td>
<td>-14±5‡</td>
<td>-17±2†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>75±7</td>
<td>67±4</td>
<td>74±3</td>
</tr>
<tr>
<td>Plasma renin activity, ng/ml/hour</td>
<td>0.17±0.03</td>
<td>0.17±0.02</td>
<td>0.16±0.06</td>
</tr>
<tr>
<td>Systemic aldosterone, pg/ml</td>
<td>684±68</td>
<td>1194±156*</td>
<td>971±139*</td>
</tr>
<tr>
<td>Endothelin-1, pg/ml</td>
<td>5.6±0.2</td>
<td>7.9±0.8†</td>
<td>5.9±0.3†</td>
</tr>
<tr>
<td>Stroke volume, ml</td>
<td>54±5</td>
<td>46±3</td>
<td>47±5</td>
</tr>
<tr>
<td>Cardiac output, Liter/min</td>
<td>3.5±0.4</td>
<td>2.8±0.1</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>E/A ratio</td>
<td>1.98±0.17</td>
<td>1.04±0.10*</td>
<td>1.47±0.15†</td>
</tr>
<tr>
<td>LV muscle mass/body weight, g/kg</td>
<td>2.14±0.16</td>
<td>3.43±0.16*</td>
<td>2.79±0.16†</td>
</tr>
</tbody>
</table>

*P<0.05 vs normal; †P<0.05 vs HTN; ‡P<0.05 vs baseline.

MAP indicates mean arterial pressure; LV, left ventricle.

lowed by an unpaired t test, and paired Student t tests detected changes within groups; P<0.05 was considered significant.

Results

Cardiac and Microvascular Function

After a 10-week observation MAP was similarly increased in HTN and HTN+bindarit compared to normal (Table, ANOVA P=0.01), indicating that bindarit did not influence blood pressure. PRA was not significantly different among the groups, whereas aldosterone levels were elevated in HTN (ANOVA P=0.03) and unaltered by bindarit (P=0.17 versus HTN+bindarit), suggesting no significant effect on the systemic renin-angiotensin- aldosterone system. In response to adenosine, MAP significantly decreased in all groups (P<0.05, Table), whereas heart rate remained unchanged. The degree of MAP response was greater in HTN compared to normal (Figure 1C). In contrast, in normal and HTN+bindarit, MAP increased in HTN (P<0.01), indicating that bindarit did not influence MAP. E/A ratio significantly decreased in HTN compared to normal (Figure 1B), suggesting LV hypertrophy, which was significantly limited (P=0.01 versus HTN) but not abolished by bindarit. There were no significant differences in stroke volume, ejection fraction, and cardiac output among the groups (P>0.05 for all), suggesting sustained cardiac systolic function. E/A ratio significantly decreased in HTN compared to normal (P=0.0006, Figure 1A), suggesting diastolic dysfunction. Importantly, E/A was increased in HTN+bindarit compared to HTN (Figure 1A, P=0.02), indicating that MCPs inhibition protected cardiac diastolic function, and was not significantly lower than normal (P=0.06).

Patlak-derived (Figure 1B) myocardial MP and FVV in the LV anterior wall were similar among the 3 groups at baseline (ANOVA P=0.60 and P=0.14, Figure 1C and 1D). In response to adenosine, MP significantly increased in HTN (from 0.0083±0.0009 to 0.0103±0.0011 AU, P=0.038 versus baseline) and achieved levels significantly higher than normal (0.0064±0.0011 AU, P=0.016 versus HTN, Figure 1C). In contrast, in normal and HTN+bindarit pigs, MP
remained unaltered during adenosine. The degree of change in MP in response to adenosine was also significantly greater in HTN compared to normal (+27±10 versus -20±5%, P=0.003). Furthermore, adenosine induced a significant decrease in FVV in HTN, which bindarit attenuated (Figure 1D). Hence, HTN-induced microvascular dysfunction was blunted by MCP inhibition.

**Microvascular Density**

There was no difference among the groups in overall transmural density of microvessels (diameters 20 to 500 μm) (ANOVA P=0.90, Figure 1F). However, subepicardial and subendocardial spatial density of larger microvessels (200 to 500 μm) was selectively decreased in HTN (all P=0.002 versus normal, Figure 1G) but preserved by MCP inhibition (Figure 1G).

**Inflammation and Remodeling**

In HTN, myocardial MCP-1 expression significantly increased mainly in the intramyocardial arterial wall, but was inhibited by bindarit (P=0.0004, Figure 2), as was perivascular macrophage accumulation (P<0.0001, Figure 2), whereas IL-6 expression in HTN remained elevated (Figure 2). COX-1 and COX-2 expression was similar among the groups. Myocardial fibrosis in HTN (ANOVA P=0.003) was significantly ameliorated (P=0.04 HTN versus HTN+bindarit), but not abolished, by bindarit (Figure 3A and 3D). Myocyte cross-sectional area increased in HTN (P=0.02 versus normal) and HTN+bindarit (P=0.02 versus normal; Figure 3B and 3E) but was not different between them (P=0.46 versus HTN), indicating no significant effect of bindarit on myocyte hypertrophy. Contrarily, microvascular media-to-lumen ratio increased from 0.08±0.01 in normal to 0.15±0.01 in HTN (P=0.006) and decreased in HTN+bindarit (to 0.10±0.02, P<0.05 versus HTN, Figure 3C and 3F), suggesting inhibition of microvascular wall thickening.

The endothelial and perivascular expression of pMYPT1 increased in both HTN and HTN+bindarit (P=0.02 and P=0.01 versus normal, respectively), suggesting that bindarit may not influence the ROCK pathway (supplemental Figure IA and ID). Endothelial ZO-1 expression was also upregulated in HTN and HTN+bindarit (supplemental Figure IB and IE, P=0.04 and P=0.03 versus normal, respectively), but significantly decreased in bindarit-treated compared to untreated HTN pigs (P=0.04, supplemental Figure IC and IF).

In addition, HTN increased both systemic and myocardial ET-1 level, which was preserved by bindarit (Table, Figure 3). AT1R expression was also elevated in HTN, but unaffected by bindarit (P=0.24, Figure 3). On the other hand, myocardial eNOS expression was slightly but significantly decreased in both HTN and HTN+bindarit (P=0.04 and P=0.02, respectively). Superoxide production by DHE was elevated in HTN (P=0.02 versus normal) and unaltered by bindarit (P=0.7 versus HTN, supplemental Figure II).
NAD(P)H oxidase p67phox expression was increased in both HTN ($P<0.01$) and HTN+bindarit ($P<0.04$), whereas p47phox remained unchanged. Therefore, bindarit did not ameliorate NAD(P)H oxidase-dependent oxidative stress (supplemental Figure II).

Cell culture studies have shown that MCP-1 dose-dependently and significantly increased collagen-I and collagen-III production and TIMP-1 expression in HCF (supplemental Figure III).

Discussion

The current study shows that specific inhibition of MCPs in hypertension ameliorated the increase in microvascular permeability and decrease in fractional vascular volume in response to increased cardiac demand, suggesting preserved microvascular integrity and endothelial barrier function. These were accompanied by attenuated microvascular remodeling and myocardial fibrosis, and by improved cardiac diastolic function. These results suggest that the myocardial and microvascular changes induced by hypertension are partly mediated by MCPs and functionally consequential.

HTN is often associated with activation of the renin-angiotensin system, ET-1, oxidative stress, and decreased nitric oxide (NO) bioavailability.$^{25,26}$ HTN is additionally characterized by inflammation and overexpression of MCPs, predominantly MCP-1,$^{27}$ a critical mediator of macrophage accumulation.$^{26}$ Activated macrophages, in turn, produce many cytokines, chemokines, growth-factors, and proteases to promote inflammation and facilitate cell proliferation, extracellular-matrix turnover, and angiogenesis. Our previous studies demonstrated that renovascular HTN increased both cardiac and renal MCP-1 protein expression and oxidative stress.$^{17,25,28}$ Furthermore, HTN altered myocardial microvascular function and impaired myocardial perfusion responses to challenge.$^{2,28,29}$ In this study we assessed several inflammatory pathways potentially activated in HTN. COX-1 and COX-2 expression remained unchanged, arguing against their involvement in myocardial alterations in our model. IL-6 increased in HTN but remained elevated in HTN+bindarit, whereas MCP-1 expression decreased in HTN+bindarit in association with improvement of many aspects of microvascular function and structure, suggesting that it might contribute to these alterations.

Hypertension induces myocyte hypertrophy, interstitial fibrosis, and consequent cardiac stiffness, which are implicated in increased LVMM and impaired diastolic function (eg, decreased E/A). In this study MCPs synthesis inhibition improved cardiac diastolic function, likely by ameliorating cardiac stiffness and increasing compliance. Interestingly, our HCF study suggests that MCP-1 can directly increase myocardial matrix production and fibrosis, thus its action is not necessarily mediated only by monocyte recruitment. Bindarit decreased interstitial fibrosis, but not myocyte hypertrophy, likely because blood pressure remained elevated, resulting in incomplete improvement of LVMM. Our observations are underscored by previous studies in rodents, in which MCP-1 was inhibited with anti–MCP-1 neutralizing antibody or

Figure 2. The expression of myocardial MCP-1, its receptor CCR2, cyclooxygenase (COX)-1, COX-2, IL-6, and macrophage infiltration in normal, hypertension (HTN), and HTN+bindarit pigs detected by immunohistochemistry. A, Representative images (magnification ×40) of MCP-1 and macrophage staining (brown, arrows), showing increase in HTN and decrease by bindarit. B, Western blots, showing increased expression of MCP-1, CCR-2, and IL-6 in both HTN and HTN+bindarit. COX-1 and COX-2 expression was similar among the groups. C, Quantitation and densitometry normalized by GAPDH. *$P<0.05$ vs normal and †$P<0.05$ vs HTN.
indirectly reduced using ROCK inhibitor, antioxidants, and inactivation of CCR-2.\textsuperscript{30--33}

Importantly, bindarit is able to modulate the levels and attenuate overproduction of MCPs in response to inflammation, without entirely blocking their physiological activity or inadvertently increasing MCPs expression, as observed with anti–MCP-1 antibody.\textsuperscript{34} In line with previous studies showing that bindarit dose-dependently inhibits MCP-1/CCL-2 production and is quite selective among chemokines,\textsuperscript{14,35} we found that in our model it did not decrease the upregulated IL-6 expression.

Interestingly, we observed that HTN increased MP and decreased FVV in response to adenosine, suggesting vascular dysfunction possibly mediated by inflammation. Hypertension initially evokes coronary endothelial dysfunction and abnormal myocardial perfusion regulation, and subsequently vascular remodeling and dysfunction.\textsuperscript{4} Endothelial barrier function is important for maintaining vascular integrity and regulation of vascular tone. MCP-1 directly increases blood-brain barrier permeability in inflammation by regulating the expression and rearranging endothelial tight junction proteins\textsuperscript{36,37} that maintain endothelial barrier integrity. We found increased expression of the endothelial tight junction component ZO-1, a membrane protein that links the actin cytoskeleton and tight junctions.\textsuperscript{38} Its endothelial overexpression in HTN may imply tight junction reassembly, a compensatory effect that may reflect disruption of endothelial barrier integrity.\textsuperscript{39} Notably, subtle changes in barrier function may be disclosed only during challenges like increased cardiac demand.\textsuperscript{40} ROCK also mediates some of the effects of MCP-1 on brain MP\textsuperscript{37} but does not seem to be a major modulator of myocardial MP in our model, because bindarit improved MP without changing MYPT (and hence ROCK activity). Nevertheless, other tight-junction proteins might contribute to loss of endothelial integrity in HTN.

Myocardial FVV decreases during cardiac challenge in HTN also indicate microvascular dysfunction, possibly consequent to microvascular wall remodeling and architectural changes.\textsuperscript{4} Indeed, microvascular rarefaction is commonly observed in HTN. We observed a selective decrease in density of larger microvessels (200 to 500 \(\mu\text{m}\)).\textsuperscript{25} The unchanged total myocardial microvascular density in HTN

\begin{figure*}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Myocardial fibrosis (A), myocyte cross sectional area (B), and vessel wall to lumen ratio (C) quantified in trichrome-, H&E-, and SMA-stained slides (magnification \(\times40\)) and myocardial expression of ET-1 and AT1R detected by Western blotting from normal, hypertension (HTN), and HTN+bindarit pigs. Myocardial fibrosis was increased in HTN compared to normal, and significantly ameliorated, but not abolished, by bindarit (D). Myocyte cross sectional area was increased in both HTN and HTN+bindarit compared to normal, but was not different between the 2 groups (E), indicating that bindarit had no effect on myocyte hypertrophy. Microvascular media-to-lumen ratio increased in HTN compared to normal and decreased in HTN+bindarit compared to HTN, yet remained higher than normal (F). Myocardial tissue ET-1 level significantly increased in HTN, which was significantly preserved by bindarit (G and H); AT1R expression was elevated in HTN, but unaffected by bindarit (G and I). *\(P<0.05\) vs normal and †\(P<0.05\) vs HTN.}
\end{figure*}
pigs may reflect an earlier increase in the number of small microvessels needed to support developing LV hypertrophy.\textsuperscript{2,4,25,28} Prevention of microvascular rarefaction by bindarit in HTN is likely through decreased myocardial and perivascular fibrosis.\textsuperscript{41} In addition, we observed in HTN increased media-to-lumen ratio, which possibly precedes luminal narrowing.\textsuperscript{23} The improvement achieved by MCPs inhibition implicates them in microvascular remodeling in HTN. Overall, MCPs inhibition decreased inflammation-induced vascular remodeling, rarefaction, and dysfunction, and blunted the reactive decrease of FVV in response to adenosine.

In addition to microvascular remodeling, myocardial vascular volume (FVV) could be affected by changes in vascular tone. Adenosine normally induces vasodilatation that involves both endothelial-dependent and -independent mechanisms.\textsuperscript{2} During endothelial dysfunction, a decrease in NO and increased abundance of vasoconstrictors (eg, ET-1) may lead to paradoxical vasoconstriction.\textsuperscript{2,29} By upregulating ET-1\textsuperscript{111} MCPs might contribute to endothelial dysfunction in HTN. This notion is underscored by our observation that MCPs blockade decreased systemic and myocardial ET-1 levels and ameliorated the decrease in FVV in response to cardiac demand. Notably, because FVV responses improved without altering eNOS expression or oxidative stress, a decrease in NO availability was unlikely a critical factor. Adenosine infusion may amplify a decrease in FVV by disclosing functional impairments, a failure in capillary recruitment, or microvascular rarefaction in HTN.\textsuperscript{42}

Systemic PRA increases shortly after induction of experimental renovascular HTN,\textsuperscript{17,18} but in the chronic phase returns to basal levels\textsuperscript{43} and may sustain HTN by activating secondary mechanisms. However, the effects of bindarit in our model were not mediated by inhibition of the renin-angiotensin-aldosterone system, which remained unchanged (PRA, myocardial AT-1R, or circulating aldosterone levels). Because MCP inhibition did not significantly blunt oxidative stress or the renin-angiotensin system, systemic hypertension remained unabated, and the beneficial effects of bindarit on cardiac function were likely derived from direct attenuation of cardiac remodeling (Figure 4). Further studies are needed to dissect the potential interaction of MCPs with the renin-angiotensin system.

Our study was limited by the use of a relatively small number of young pigs. We also used in all groups 2 fast-CT scanners, which provide comparable assessments of MP and FVV.\textsuperscript{7} It is possible that some of the tissue studies sampled some unbound circulating proteins, but exanguinated myocardium contains little blood volume, and the preparation procedure was the same for all the groups. Therefore, the fraction of retained unbound cytokines might be negligible and comparable among the groups, and result in minimal interference with our result interpretation. Bindarit inhibits MCPs 1, 2, and 3, but MCP-1 is the key member of the MCP family that has been most commonly and directly linked to cardiovascular disease and hypertension, so that the effects of the drug are likely attributable mainly to blockade of MCP-1. Nevertheless, we cannot exclude the possibility that inhibition of MCPs 2 and 3 contributed to these benefits. Addition-


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An erratum has been published regarding this article. Please see the attached page for:
/content/30/12/e323.full.pdf
In the article, “Monocyte Chemoattractant Proteins Mediate Myocardial Microvascular Dysfunction in Swine Renovascular Hypertension” by Lin et al, which appeared in the November 2009 issue of the journal (Arterioscler Thromb Vasc Biol. 2010;29:1810–1816; DOI: 10.1161/ATVBAHA.109.190546), there were a few errors in the final, printed version of the article:

1. Page 1811, the correct table caption is “Systemic and Cardiac Function (mean±SEM) in Normal, Hypertension (HTN), and HTN+Bindarit Pigs”
2. Page 1811, 1st column, line 3, the supplier of female domestic pigs is “Park Partners”

The online version has been corrected.

The publisher sincerely regrets the errors.

DOI: 10.1161/ATV.0b013e318203abc2
Animal Studies

Methods

In-vivo CT Studies

To evaluate cardiac and microvascular function in vivo, pigs were scanned by fast CT, either electron beam computed tomography (EBCT, C-150, Imatron, South San Francisco, CA) or 64-slice multidetector CT (MDCT, SOMATOM Sensation 64, Siemens Medical Solutions, Forchheim, Germany) as previously described.\(^1\) We have previously shown that these scanners provide very similar assessments of MP and FVV.\(^1\)

For fast CT, using localization scans, mid LV levels were selected for measurement of microvascular function. A 50 second flow study was then performed during respiratory suspension at end-expiration immediately following a bolus injection of the nonionic, low osmolar contrast medium iopamidol (Isovue-370, 0.33 mL/kg over 2 seconds) into the right atrium. After a 15-minute rest, the functional study was repeated during a 5-minute intravenous infusion of adenosine (400 µg/kg/min). With EBCT (n=2 of each group), the multislice flow mode was employed to obtain two parallel 8-mm thick tomographic sections in a scan time of 50 milliseconds. Scanning was triggered at the end-diastolic phase, and 40 images were obtained at 1–3 beat intervals, as we have previously described.\(^2-5\) With MDCT (n=4 of each group), two parallel 6-mm thick sections of the heart were studied throughout the cardiac cycle using a full scan reconstruction (330 milliseconds) with a scan reconstruction increment of 50 milliseconds.\(^1\) For measurements of cardiac systolic function, diastolic filling...
function, and LVMM, the entire LV was scanned using 16-20 time points throughout the cardiac cycle to obtain multi-levels heart images.

**CT Data Analysis**

All images were analyzed with the Analyze software package (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN). For LV size and systolic function, the LV endocardial and epicardial surfaces were traced at end-diastole and end-systole. LV ejection fraction, stroke volume, cardiac output, and LVMM were calculated as previously described.\(^5\) For diastolic function, the total volume of the LV cavity was calculated throughout the cardiac cycle, and the data then plotted as time-volume curves. Early (E) and late (A) LV filling rate were calculated from the positive slopes of the curve, as described before.\(^5,\,6\)

For microvascular function, regions of interest in the LV and anterior wall were manually traced at end-diastole.\(^1-5,\,7\) The time-density data were then used to assess MP (arbitrary unit (AU)) and FVV (%) based on a Patlak graphical analysis method, as previously described.\(^1,\,8,\,9\) The Patlak method is a unidirectional simplified 2-compartment (intravascular and extravascular space) model that derives MP and FVV with a linear regression according to the formula:

\[
\frac{Myo}{LV} = FVV + MP \cdot \int_0^t LV(\tau) \cdot d\tau 
\]

*Myo* and *LV* represent myocardial and LV cavity CT numbers (opacity) after injecting contrast media, respectively, and *t* is time (sec). The change in myocardial opacity corresponds to transit of the contrast medium bolus in the intravascular and extravascular compartment of the myocardium.\(^1,\,10\) The Y-
Intercept of the Patlak plot represents the FVV, and the slope is representative of the MP (extravascular contrast media accumulation per unit volume).\textsuperscript{1}

**Micro-CT Study**

To evaluate myocardial microvascular density, micro CT scanning was performed, as previously described\textsuperscript{11,12}. Briefly, after the heart was excised and perfused with microfil silicone rubber (MV-122, Flow Tech, Inc) through a left anterior descending artery cannula under physiological pressure, and an anterior wall myocardium segment (\( \approx 2 \times 1 \times 1 \text{ cm} \)) was dissected and scanned at 0.49\textdegree increments. Myocardial images were tomographically divided into subepicardium and subendocardium,\textsuperscript{11,12} and the spatial density of myocardial microvessels (diameters <500 \( \mu \text{m} \)) calculated in each region (rendering threshold was set equally for all the images).\textsuperscript{11}

**Histology**

*Myocardial remodeling.* LV myocardial tissue was fixed in formalin, embedded in paraffin, and 5 \( \mu \text{m} \) sections were then cut and stained. In H&E stained slides, 5 regions were randomly selected in each slide (magnification of X40). Using the MetaMorph software (Mata Image Series 6.3), the cross sectional areas of 10 round myocytes with central nuclei were measured.\textsuperscript{13} In addition, in one trichrome-stained slide from each pig (magnification of X40), 3 fields were randomly selected and the fraction of blue staining relative to the background was measured.

*Microvascular remodeling.* Three regions including intramyocardial microvessels were randomly selected from slides (X40 magnification) stained
using anti-human α-smooth muscle actin (SMA) (DakoCytomation) antibody. Microvascular wall thickness and lumen diameter were then measured and their ratio calculated.¹⁴

**Immunohistochemistry**

To assess indices of inflammation and MP, the primary antibodies rabbit anti-MCP-1 (1:50, Biovision), mouse anti-macrophage CD163 (1:20, Serotec), rabbit anti-zonula occludens-1 (ZO-1, 1:50, Zymed), and rabbit anti-phospho-myosin phosphatase targeting subunit (Thr⁶⁹⁶-pMYPT1, 1:50, Upstate) were incubated with deparaffined and rehydrated slides and detected by the avidin-biotin complex technique using the DAB chromogen (Vector) with hematoxylin counterstain. MYPT1 was detected by Texas-red conjugated secondary antibody. Quantitative analysis was achieved using MetaMorph to calculate the fraction of positive stain for three random fields per slide.

**Western Blotting**

Total proteins from heart lysates (100 µg) were loaded onto 4-15% SDS-polyacrylamide gel (Bio-Rad) and transferred onto PVDF membrane (Millipore). The following primary antibodies (rabbit IgG) were used for blotting: anti-MCP-1 (1:5000, Mybiosource), anti-CCR2 (1:500, ABR Affinity), anti-IL-6 (1:500, Millipore), anti-COX1 (1:500, Abcam), anti-COX-2 (1:500, Lifespan), anti-AT1R (1:200, Santa Cruz), anti-ET-1 (1:200, Santa Cruz), anti-endothelial nitric oxide synthase (eNOS, 1:200, Santa Cruz), anti-p47 and p67 (both 1:1000, Santa Cruz), and anti-ZO-1 (1:1000, Zymed). GAPDH was blotted to confirm equal loading. A goat anti-rabbit secondary antibody was used at 1:2000 dilution.
Chemiluminescence (Pico Substrate Chemiluminescence kit, Pierce) was used for detection. Densitometry values were normalized to GAPDH and expressed as ratio.

**In Vitro Cell studies**

**Methods**

**Cell Culture and Treatments**

Human cardiac fibroblasts (HCFs, Cell Applications) were cultured in cardiac fibroblast culture media (Cell Applications), in a humidified incubator at 37°C with 5% CO₂. HCFs were starved in Opti-MEM for 16 hours and then incubated with MCP-1 (R&D Systems, 0, 1, 5, 10, and 20ng/ml) for 24 hours in the cell culture incubator. Cells pellets were subsequently harvest for western blotting.

**Western Blotting**

Equal amount protein (50 µg) from the cells were loaded onto 4-15% SDS-polyacrylamide gel (Bio-Rad) and transferred onto PVDF membrane (Millipore). The following primary rabbit IgG: anti-Collagen I (1:500, COSMO Bio), and anti-TIMP (1:200, Santa Cruz), and mouse IgG: anti-Collagen III (1:1000, Millipore) were used for blotting. GAPDH was blotted to confirm equal loading. Goat anti-rabbit and donkey anti-mouse secondary antibody was used at 1:2000 dilution. Chemiluminescence (Pico Substrate Chemiluminescence kit, Pierce) was used for detection. Densitometry values were normalized to GAPDH and expressed as ratio.
Results

In HCFs, we found that incubation with MCP-1 dose-dependently and significantly increased the production of both collagen I and collagen III, as well as the expression of TIMP-1 (Figure 3S). These observations suggest that MCP-1 can have a direct action in the myocardium to increase matrix production, and thus fibrosis, and that this action is not necessarily mediated by recruitment of monocytes or by macrophages. This important piece of evidence therefore ascribes a direct role to MCP-1 in myocardial remodeling. Nevertheless, in all likelihood monocyte/macrophage also contribute greatly to the inflammation responses inflicted by MCP-1.
References


Figures Legends

Figure 1S. Phospho-myosin phosphatase targeting subunit (pMYPT1, A) and ZO-1 (B and C) expression detected in the myocardium by fluorescent immunostaining (Texas-Red), colorimetric immunostaining and Western blot, respectively, and quantified (D, E and F) at a magnification of X40. The endothelial and perivascular expression of pMYPT1 was elevated in the HTN and HTN+bindarit. ZO-1 endothelial expression was upregulated in HTN and HTN+bindarit compared to normal. * p<0.05 vs. normal and † p<0.05 vs. HTN.

Figure 2S. Myocardial expression of eNOS and NAD(P)H oxidase (p47 and p67) detected with Western blotting (A) and superoxide production detected by dihydroethidium (DHE) staining (B) in normal, hypertension (HTN), and HTN+bindarit pigs. Densitometry was normalized by GAPDH and DHE staining by nuclei (C). The expression of eNOS was similarly and significantly decreased in both HTN and HTN+bindarit. NAD(P)H oxidase (p67 subunit) was increased, while p47 did not differ among the groups. Superoxide production was elevated in HTN and unaltered by bindarit. *p<0.05 vs. normal and † p<0.05 vs. HTN.

Figure 3S. The effects of MCP-1 on human cardiac fibroblasts (HCFs). A. Western blots, showing the expression of collagen I, collagen III, and TIMP-1 in HCFs treated with different concentration of MCP-1. B. Densitometry normalized by GAPDH. MCP-1 dose-dependently and significantly increased the production
of both collagen I and collagen III, as well as the expression of TIMP-1. * p<0.05 vs. control (MCP-1 concentration is 0 ng/ml).
Figure 1S
Figure 2S
**Figure 3S**

### A

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### B

- **Collagen I**
  - Density / GAPDH

- **Collagen III**
  - Density / GAPDH

- **TIMP-1**
  - Density / GAPDH