Induction of Mitogen-Activated Protein Kinases Is Proportional to the Amount of Pressure Overload

Giovanni Esposito, Cinzia Perrino, Gabriele Giacomo Schiattarella, Lorena Belardo, Elisa di Pietro, Anna Franzone, Giuliana Capretti, Giuseppe Gargiulo, Gianluigi Pironti, Alessandro Cannavo, Anna Sannino, Raffaele Izzo, Massimo Chiariello

Abstract—Pressure overload has been shown to induce mitogen activated protein kinases (MAPKs) and reactivate the atrial natriuretic factor in the heart. To test the sensitivity of these signals to pressure overload, we assayed the activity of MAPKs extracellular signal–regulated kinase, c-Jun N-terminal kinase 1, and p38 in protein lysates from the left ventricle (LV) or white blood cells (WBC) isolated from aortic banded mice with varying levels of pressure overload. In separated mice we measured atrial natriuretic factor mRNA levels by Northern blotting. As expected, a significant induction of atrial natriuretic factor mRNA levels was observed after aortic banding, and it significantly correlated with the trans-stenotic systolic pressure gradient but not with the LV weight:body weight ratio. In contrast, a significant correlation with systolic pressure gradient or LV weight:body weight ratio was observed for all of the MAPK activity detected in LV samples or WBCs. Importantly, LV activation of MAPKs significantly correlated with their activation in WBCs from the same animal. To test whether MAPK activation in WBCs might reflect uncontrolled blood pressure levels in humans, we assayed extracellular signal–regulated kinase, c-Jun N-terminal kinase 1, and p38 activation in WBCs isolated from normotensive volunteers, hypertensive patients with controlled blood pressure values, or hypertensive patients with uncontrolled blood pressure values. Interestingly, in hypertensive patients with controlled blood pressure values, LV mass and extracellular signal–regulated kinase phosphorylation were significantly reduced compared with those in hypertensive patients with uncontrolled blood pressure values. These results suggest that MAPKs are sensors of pressure overload and that extracellular signal–regulated kinase activation in WBCs might be used as a novel surrogate biomarker of uncontrolled human hypertension. (Hypertension. 2010;55:137-143.)

Key Words: hypertension ■ leukocytes ■ cardiac hypertrophy ■ MAPKs ■ ERK

Human arterial hypertension is a common and largely asymptomatic disease. Uncontrolled values of blood pressure significantly increase the risk of serious adverse cardiovascular events, thereby accounting for a significant cost burden. The effective reduction of blood pressure levels has been shown to reduce major cardiovascular events.

An intricate network of intracellular signaling pathways is induced by pressure overload in cardiomyocytes. Indeed, normal and hypertrophied hearts exhibit significant qualitative, as well as quantitative, differences in the activation of molecular signaling pathways and gene expression. However, the role of antihypertensive agents on intracellular signaling pathways is not well known.

One of the best-described signal transmission systems activated by pressure overload involves multiple cascades of protein phosphorylation by the mitogen activated protein kinase (MAPK) family. The MAPK superfamily includes 3 principal protein members: extracellular signal–regulated kinase (ERK), c-Jun N-terminal kinase 1 (JNK1), and p38, which are involved in different cellular processes, including pressure overload–induced cardiac hypertrophy. Whether the activation of these signaling pathways is correlated to the amount of pressure overload and, importantly, whether they might be used as molecular sensors of cardiac stress are still unclear.

A number of genes have been identified that exhibit altered expression patterns in cardiac hypertrophy, including the atrial natriuretic factor (ANF). Ventricular expression of ANF increases with conditions of increased hemodynamic load often associated with increases in ventricular mass. However, we have shown recently that a pathological cardiac phenotype can be dissociated from gene expression reprogramming in mice, and, therefore, whether altered expres-
sion of ANF can be used as a marker of pressure overload is controversial.

In the present study, we examined the relationship between the amount of cardiac pressure overload and the activation of molecular signaling pathways in the heart. Our results indicate that MAPK activation has a strong correlation with the amount of pressure overload. Because recent studies suggest that signaling properties in peripheral blood cells may mirror similar changes in the heart,7 we next tested whether MAPK activation in white blood cells (WBCs) might be used as a novel biomarker of uncontrolled human hypertension.

Materials and Methods
An expanded section on Materials and Methods can be found in the online Data Supplement (please see http://hyper.ahajournals.org).

Results
Effect of Incremental Pressure Overload on Cardiac Hypertrophy and ANF Re-Expression
To test the impact of incremental pressure overloads on the activation of intracellular signaling pathways, we analyzed a large group of aortic banded mice with varying levels of load, ranging from a trans-stenotic gradient of 5 to \( \approx 150 \) mm Hg. As expected, as a group, transverse aortic constriction (TAC) mice exhibited increased cardiac mass compared with sham mice, expressed by an increased left ventricle weight/body weight (LVW/BW) ratio (sham: 3.3\( \pm \)0.08, \( n=12 \); TAC: 4.02\( \pm \)0.15, \( n=16 \); \( P<0.01 \)). Interestingly, over a wide range of pressure gradients analyzed, we found a significant correlation between the systolic pressure gradient and the amount of cardiac hypertrophy developed (\( R^2=0.9; P<0.001 \)). These results indicate that the increase in cardiac mass is proportional to the amount of pressure overload.

In response to cardiac pressure overload, the ANF gene is re-expressed and is considered a well-established marker of pathological hypertrophy.8 Because cardiac hypertrophy is fully established after 7 days of pressure overload (for the supplementary figure, please see Figure S1A in the online Data Supplement) and the maximum ANF levels recorded were achieved at this time point (Figure S1B), we tested the correlation between pressure overload and ANF expression by Northern blotting in sham and TAC mice 7 days after the surgery. As expected, TAC mice exhibited increased ANF expression compared with sham mice (Figure 1A), and ANF levels were significantly correlated to the systolic pressure gradient (SPG; Figure 1B). However, the linear correlation between ANF levels and LVW/BW ratios was not significant (Figure 1C).

MAPK Activation Is a Sensitive Marker of Cardiac Pressure Overload
We have demonstrated previously that MAPKs are early activated in response to pressure overload and that 7 days after the TAC ERK (Figure S1C), JNK1, and p38 are fully activated.9 Thus, we measured MAPK activation in protein lysates of LV samples or WBCs isolated from sham and TAC mouse hearts explanted 7 days after banding. Consistent with previous results, ERK kinase activity was significantly induced by pressure overload in the heart (Figure 2A). Importantly, ERK activation was also significantly increased in WBCs isolated from the same animals (Figure 2A).
Over a wide range of pressure gradients, LV ERK activation had a strong linear correlation with the amount of pressure overload as measured by SPG (Figure 2B), and it was also linked to cardiac hypertrophy, as measured by an increased LVW/BW ratio (Figure 2C). Interestingly, ERK activation in WBCs isolated from sham and TAC mice also displayed a strong linear correlation with the SPG (Figure 2D), and it was also strongly linked to the LVW/BW ratio (Figure 2E). Indeed, ERK activation in LV samples was significantly correlated to ERK activation in WBCs (Figure 2F).

Similar results were also obtained for the MAPKs JNK1 (Figure 3) and p38 (Figure 4). For all of the MAPKs analyzed, we did not observe significant changes of protein levels evaluated by immunoblotting (data not shown), suggesting that, at the time point assayed (7 days of TAC), the activation of MAPKs is solely attributable to induction of the protein kinase activity. Taken together, our results suggest that these molecular signaling pathways are sensitive to pressure overloads and are tightly linked to the development of cardiac hypertrophy.

Increased ERK Phosphorylation in Peripheral Blood Leukocytes From Patients With Uncontrolled Blood Pressure Levels

To assess the correlation between pressure overload and the activation of molecular signaling pathways in circulating human WBCs, we included in this study patients with essential hypertension not sufficiently controlled by pharmacological treatment (UHT), patients with essential hypertension sufficiently controlled by pharmacological treatment (CHT), and healthy volunteers (CON). As expected, before pharmacological therapy, unadjusted mean LV mass in hypertension patients was significantly higher compared with the control group (data not shown). Eighteen months after pharmacological therapy for hypertension, LV mass was significantly reduced in CHT patients compared with the UHT group (Figure 5A).

It is well known that β-adrenergic receptors signaling is dysfunctional in hearts exposed to increased pressure loads. To assess a possible alteration of β-adrenergic receptors signaling in human leukocytes induced by arterial hypertension, we assessed G protein–coupled receptor kinase 2 (GRK2) levels in WBCs from CON and hyp-
tensive subjects, either with normal blood pressure levels (CHT) or uncontrolled hypertension (UHT). As shown in Figure 5B, GRK2 expression levels were significantly increased in normal subjects compared with hypertensive patients ($P<0.05$); importantly, UHT patients exhibited significantly higher GRK2 levels compared with CHT patients ($P<0.05$). Consistent with these results, GRK2 levels were also increased in leukocytes isolated from TAC mice (Figure S2).

We next tested whether uncontrolled values of arterial blood pressure might affect MAPK signaling in WBCs. Although ERK protein levels in leukocytes did not change among the different groups, a significant increase in ERK phosphorylation was selectively found in WBCs from patients with elevated blood pressure levels (UHT) compared with normotensive subjects (Figure 5C; ERK fold activation in UHT samples: mean, 2.4; SD, ±0.8; SE, ±0.17). Interestingly, CHT patients displayed a significantly reduced ERK activation (Figure 5C; ERK fold activation in CHT samples: mean, 1.3; SD, ±0.6; SE, ±0.13). Moreover, a significant correlation was found between ERK activation in WBCs and systolic arterial blood pressure >150 mm Hg (Figure S3). Importantly, increased ERK phosphorylation was very consistent and reproducible among the different samples within the same group, as shown by the low SD and SE in the different groups of patients.

Similar to ERK, JNK1 activation was significantly induced in UHT patients (Figure 5D); however, effective antihypertensive pharmacological therapy did not exert any significant effects on JNK1 activation in CHT patients (Figure 5D). In contrast to other MAPKs, p38 activation in human WBCs isolated from HT patients was not statistically different from CON subjects (Figure 5E). Taken together, these studies suggest that increased ERK activation in WBCs might identify a subgroup of hypertensive patients with uncontrolled domiciliary blood pressure levels.

**Discussion**

In the present study, we investigated the correlation between the amount of pressure overload and the activation of stress-induced molecular signals in the heart and WBCs. MAPK activity in LV samples and WBCs from TAC mice significantly correlated with the amount of pressure overload and cardiac hypertrophy development. Furthermore, UHT patients displayed a significant activation of ERK signaling in WBCs compared with healthy volunteers. Importantly, in CHT patients, LV mass and WBC ERK signaling were significantly reduced. These data support the important role of MAPKs as molecular sensors and signal transducers of elevated blood pressure levels and suggest that the evaluation of ERK signaling in
WBCs might represent a novel important tool to establish the effectiveness of pharmacological treatment in hypertensive patients.

Recent studies have shown the important role of MAPKs in several human cardiovascular diseases. We have shown previously the early activation of the MAPKs in mouse models of left ventricular hypertrophy and in human left ventricular samples during cardiopulmonary bypass. In contrast to ANF re-expression, activation of MAPKs ERK, JNK1, and p38 had a strong correlation with the trans-stenotic pressure gradient measured and the LVW/BW ratio developed in individual mice over a wide pressure range, suggesting that MAPKs might be considered molecular sensors of pressure overload in the heart.

In this study we tested whether ERK activation in WBCs might reflect pressure overload in TAC mice or blood pressure control in hypertensive patients under medical therapy. In mice exposed to increased pressure overload, ERK activation in the heart significantly correlated with ERK activation in leukocytes. Consistent with these results, leukocytes for hypertensive patients with elevated blood pressure values despite medical therapy exhibited a marked significant increase in ERK phosphorylation.

Although in the mouse TAC model of pressure overload all of the MAPKs were significantly activated both the left ventricle and in WBCs, in WBCs from patients with systolic blood pressure >150 mm Hg, ERK activation was the only molecular parameter significantly increased. The reason(s) for the preferential activation of ERK in human WBCs from hypertensive patients is currently unknown. Because the amount of pressure overload in the TAC model is presumably higher than the pressure overload detectable in UHT patients, it is possible to speculate that ERK represents a more sensitive marker of pressure overload in hypertensive patients.

The precise mechanism(s) underlying the regulation of ERK activation in human WBCs is still not completely known. In our study, hypertensive patients were characterized by increased GRK2 levels in WBCs compared with CON subjects. Consistent with these results, GRK2 levels in WBCs from TAC mice were also increased, suggesting that a hyperadrenergic state induced by pressure overload might similarly affect cardiac and peripheral β-adrenergic
receptors signaling, promoting β-adrenergic receptors dysfunction. However, we did not detect significant differences in the urinary levels of catecholamines in hypertensive patients (Table). Future studies will be needed to better address these issues.

Although it is possible to speculate that blood pressure–lowering agents might reduce ERK activation in WBCs by reducing vascular or systemic inflammation, we did not detect any differences in the urinary levels of catecholamines in hypertensive patients (Table). Future studies will be needed to better address these issues.

In conclusion, integrating the results in mice and human leukocytes, this study is evidence that MAPKs are sensors of cardiac pressure overload and that leukocytes might represent important cellular targets to mirror cardiac signaling.

**Perspectives**

In the present study, we investigated the correlation between the amount of pressure overload on mouse hearts and the activation of stress-induced molecular signals in the heart and WBCs from the same animal. Our findings suggest that MAPKs are sensors of cardiac pressure overload and that leukocytes might represent important cellular targets to mirror cardiac signaling. This study examines the implications of this new paradigm in hypertension, focusing on the opportunity to discover and characterize novel molecular biomarkers to monitor the effects of pressure overload. In particular, this study adds new information about the potential role of ERK activation in WBCs as a novel molecular marker to identify uncontrolled human hypertension. The mechanism(s) underlying this phenomenon remains uncertain. Although it is possible to speculate that pressure overload, per se, might...
activate signaling pathways in WBCs as it does in the heart, future studies will be needed to address this complicated issue.

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Disclosures
None.

References

Table. Clinical Characteristics of the Groups of Patients

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<th>CON (n=13)</th>
<th>CHT (n=38)</th>
<th>UHT (n=29)</th>
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<td>57.8±1.9</td>
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<td>SBP, mm Hg</td>
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<td>DBP, mm Hg</td>
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<td>Catecholamines, μg/24 h</td>
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<td>LV mass index, g/m²</td>
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<td>ARBs, % of patients</td>
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</table>

BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CRP, C-reactive protein; LV mass index, left ventricular mass index; ACEIs, angiotensin-converting enzyme inhibitors; DHP-CCBs, dihydropyridine calcium channel blockers; ARBs, angiotensin ii receptor blockers.

*P<0.05 vs CON and CHT subjects.
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THE AMOUNT OF PRESSURE OVERLOAD

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Short title: MAPKs and pressure overload

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Materials and Methods

Animal model of pressure overload-induced cardiac hypertrophy

The animals in this study were treated according to the procedures approved by the appointed committee of Federico II University, Naples Italy. Cardiac hypertrophy was induced in 16-week-old male wild type mice (C57BL/6) by transverse aortic constriction (TAC) as previously described. Control mice underwent the same operation but without aortic banding (sham). 7 days after the TAC procedure, the animals were anaesthetized and the transtenotic systolic pressure gradient (SPG) was invasively measured as previously described. The animals were then sacrificed, cardiac chambers dissected, weighed and frozen in liquid nitrogen.

RNA extraction and Northern Blotting

Northern Blotting analysis was performed as previously described.

MAPKs activity assay

The activity of the MAPKs ERK, JNK1 and p38 was evaluated using 2 mg of clarified extracts of the left ventricle as previously described.

Patients included in the study

Patients included in the study were divided into three groups (Table 1): 1) patients with essential hypertension not sufficiently controlled by pharmacological treatment (PA>140/80 mmHg, UHT); 2) patients with essential hypertension sufficiently controlled by pharmacological treatment (PA<140/80 mmHg, CHT); 3) healthy volunteers with normal blood pressure levels without medications (PA<140/80 mmHg, CON). Subjects with clinical history of allergic diseases or increased WBC count were excluded from the study. All participants gave written informed consent to the study, which was approved by our institutional ethics committee.

LV mass quantification by echocardiography

LV internal dimension (LVID), interventricular septal thickness (IVST), and posterior wall thickness (PWT) were all measured at end-diastole (d) with the leading-edge technique. Relative wall thickness (RWT) was calculated as RWT = (IVST + PWT)/LVID. Estimates of LV mass were calculated according to the American Society of Echocardiography criteria: LV mass (g) = 1.05[(LVIDd+IVSTd+PWTd)3−(LVIDd)3]4.

Leukocytes extraction and sample preparation

Mononuclear leukocytes were separated from EDTA anticoagulated whole blood with the method of Boyum as described previously. For the preparation of cytosolic fractions, mononuclear leukocytes were resuspended in ice-cold lysis buffer (20 mM Tris-HCl, 2 mM EDTA, pH 7.5, at room temperature, 100 µg/ml PMSF, 10 µg/ml benzamidine, and 5 µg/ml pepstatin A). Cells were subjected to nitrogen cavitation (Parr Bomb, 600 psi, 15 min at 4 °C), followed by centrifugation at 45,000 g for 30 min at 4 °C. The supernatants were collected and frozen in liquid nitrogen. Additionally, whole cell samples...
were pelleted and frozen at −80 °C.

**Immunoblotting**

Protein levels of ERK, phosphoERK and GRK2 were obtained using total protein extracts with polyclonal antibodies (Santa Cruz Biotechnology). The proteins were highlighted by means of chemiluminescence reaction with secondary antibodies conjugated with peroxidase (ECL, Amersham Pharmacia Biotech).

**Bio-Plex phosphoprotein assay**

JNK and p38 activation was tested in mouse cardiac samples and human protein lysates from CON, CHT and UHT patients by Bio-Plex phosphoprotein assay (Bio-Rad) according to the manufacturer’s instructions.

**Statistical analysis**

Data are expressed as mean ± SE. Multi-group comparisons were made with a 1-way ANOVA with a Tukey’s finishing test. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software version 4.0). For all analyses, a minimum value of $p<0.05$ was considered significant; when present, a $p$ value <0.0001 was specified.
References


Figure S1

A. LV weight/body weight (LVW/BW) ratios measured at study termination in sham and TAC mice (*p<0.05 vs. sham).

B. ANF mRNA levels (fold over sham) after 3 h, 6 h, 12 h, 2 and 7 days of pressure overload (*p<0.05 vs. sham).

C. The upper panel shows representative immunoblotting for phosphoERK (p-ERK) in left ventricular samples (LV) isolated from sham and TAC mice at different time points after pressure overload. The lower panel shows the cumulative data of multiple independent experiments on LV ERK activation (fold over sham); *p<0.05 vs. sham.
Figure S2

The upper panel shows representative immunoblotting for GRK2 levels in white blood cells (WBC) isolated from sham and TAC mice after 3 h, 6 h, 12 h, 2 and 7 days of pressure overload. The lower panel shows the cumulative data of multiple independent experiments on WBC GRK2 activation (fold over sham); *p<0.05 vs. sham.
A. Bar graphs showing cumulative data of multiple independent experiments on ERK activation (fold over CON) in white blood cells (WBC) isolated from healthy volunteers (CON), hypertensive patients with normal blood pressure levels (CHT) or uncontrolled blood pressure levels (UHT) with systolic blood pressure (SBP) > 150 mmHg or < 150 mmHg; *p<0.05 vs. all.

B. Bar graphs showing cumulative data of multiple independent experiments on ERK activation (fold over CON) in WBC isolated from CON, CHT or UHT with diastolic blood pressure (DBP) > 90 mmHg or < 90 mmHg.

C. Bar graphs showing cumulative data of multiple independent experiments on JNK1 activation (fold over CON) in WBC isolated from CON, CHT or UHT with systolic blood pressure (SBP) > 150 mmHg or < 150 mmHg.

D. Bar graphs showing cumulative data of multiple independent experiments on JNK1 activation (fold over CON) in WBC isolated from CON, CHT or UHT with diastolic blood pressure (DBP) > 90 mmHg or < 90 mmHg.

E. Bar graphs showing cumulative data of multiple independent experiments on p38 activation (fold over CON) in WBC isolated from CON, CHT or UHT with systolic blood pressure (SBP) > 150 mmHg or < 150 mmHg.

F. Bar graphs showing cumulative data of multiple independent experiments on p38 activation (fold over CON) in WBC isolated from CON, CHT or UHT with diastolic blood pressure (DBP) > 90 mmHg or < 90 mmHg.
Figure S4

A. TNF-alpha mRNA levels (fold over sham) after 3 h, 6 h, 12 h, 2 and 7 days of pressure overload in white blood cells (WBC); *p<0.05 vs. sham.

B. Cumulative data of multiple independent experiments on left ventricular samples (LV) TNF-alpha activation by Bioplex assay (fold over sham) from sham and TAC mice in response to 2 and 7 days of pressure overload; *p<0.05 vs. sham.

C. IL-1beta mRNA levels (fold over sham) after 3 h, 6 h, 12 h, 2 and 7 days of pressure overload in WBC; *p<0.05 vs. sham.

D. Cumulative data of multiple independent experiments on LV IL-1beta activation by Bioplex assay (fold over sham) from sham and TAC mice in response to 2 and 7 days of pressure overload; *p<0.05 vs. sham.

E. IL-6 mRNA levels (fold over sham) after 3 h, 6 h, 12 h, 2 and 7 days of pressure overload in WBC.

F. Cumulative data of multiple independent experiments on LV IL-6 activation by Bioplex assay (fold over sham) from sham and TAC mice in response to 2 and 7 days of pressure overload; *p<0.05 vs. sham.