Sustained activation of nuclear factor kappa B and activator protein 1 in chronic heart failure

Stefan Frantz, Daniela Fraccarollo, Helga Wagner, Thomas M. Behr, Philip Jung, Christiane E. Angermann, Georg Ertl, Johann Bauersachs

Medizinische Universitätsklinik Würzburg, Josef-Schneider-Str. 2, 97080 Würzburg, Germany
Medizinische Poliklinik der Universität Würzburg, Würzburg, Germany

Received 25 July 2002; accepted 10 October 2002

Abstract

Objective: Innate immune response proteins such as inflammatory cytokines, inducible nitric oxide synthase, and toll like receptors are implicated in myocardial depression and left ventricular (LV) remodeling after myocardial infarction (MI). Although all these innate immunity proteins share the downstream activation of the transcription factor NF-κB (nuclear factor kappa B) and activator protein 1 (AP-1), the involvement of NF-κB and AP-1 in LV remodeling has not been demonstrated so far. Methods and results: Nuclear translocation of NF-κB and AP-1 was studied by electrophoretic mobility shift assays and ELISA 10 weeks after large experimental MI in rats, the chronic phase of LV remodeling. In the non-infarcted myocardium of MI rats, NF-κB and AP-1 were significantly activated (2.5-fold) as compared to sham-operated animals. Immunohistochemistry demonstrated NF-κB activation mainly in cardiac myocytes. Treatment with the ACE (angiotensin converting enzyme) inhibitor trandolapril led to a further 2-fold increase in the activation of NF-κB and AP-1 when compared to placebo-treated animals with the same MI size (P<0.001). Human failing hearts explanted at the time of heart transplantation exhibited marked nuclear translocation of NF-κB in cardiac myocytes when compared to control hearts. NF-κB as well as AP-1 were both significantly activated in congestive heart failure due to ischemic or dilated cardiomyopathy. Conclusion: In experimental and human heart failure, both NF-κB and AP-1 are chronically activated in cardiac myocytes. These findings suggest an important involvement of NF-κB and AP-1 in the cardiac remodeling process.

Keywords: Cytokines; Immunology; Infarction; Remodeling

1. Introduction

The Rel/NF-κB (nuclear factor kappa B) transcription factor is a family of evolutionary conserved proteins that are all related through a highly conserved binding domain, the Rel homology domain [1]. Two classes of Rel proteins exist: the first class contains a long C-terminal domain that is activated by proteolysis (p105 to p50, p100 to p52) or arrested translation. In general, this group is not an activator of transcription unless dimers are formed with members of the second class. The second class includes proteins like c-Rel and RelA (p65). They contain C-terminal activation domains. All vertebrate Rel proteins can build homo- or heterodimers (except RelB). However, NF-κB usually refers to a p50–p65 (RelA) dinner which is the major Rel complex in most cells and organs including the heart [2].

The activity of NF-κB in the cell is usually tightly controlled. In the cytoplasm NF-κB interacts with its inhibitory protein IκB. Several IκB proteins exist that have different affinities to individual NF-κB complexes. The interaction of NF-κB and IκB blocks the nuclear translo-
tion and binding of DNA by NF-κB. However, upon activation of IKK (IkB kinase), IkB is phosphorylated [3], leading to a dissociation of NF-κB and IkB, as well as an ubiquitination of IkB and consequently degradation of IkB by proteasomes. Once dissociated, NF-κB can enter the nucleus and activate target gene transcription (see Ref. [4] for review).

Over 150 stimuli are known today to rapidly activate NF-κB [5]. Most of them are involved in innate immune responses. On the other hand, NF-κB activation controls the expression of over 150 target genes [5]. Most of them do also participate in host response including 27 different cytokines and chemokines, as well as receptors required for immune recognition such as toll like receptors (TLRs) [6]. However, NF-κB is also involved in the regulation of genes that regulate cell structure and wound healing like vimentin, laminin, collagenases and gelatinases [5].

Another important transcription factor of immediate to early stress response genes is activator protein 1 (AP-1). It is composed of members of the Jun and Fos families. Upon phosphorylation of Fos by the MAPK (mitogen activated protein kinase) FRK (fyn-related kinase) or phosphorylation of Jun by the MAPK JNK, a Jun kinase, AP-1 is activated [7].

The activation of a number of inflammatory cytokines, including TNF (tumor necrosis factor), IL-1β (interleukin), IL-6 and IL-8, as well as iNOS (inducible nitric oxide synthase) and TLR expression was reported in experimental and human heart failure regardless of etiology [8]. However, all of these downstream of the NF-κB and AP-1 as well as NF-κB response elements in their promoters. Although most of these innate immunity proteins have been implicated in the pathophysiology of congestive heart failure (CHF), the activation of NF-κB and AP-1 in CHF has not yet been systematically demonstrated. We therefore sought to determine the activation of NF-κB and AP-1 in an experimental model of chronic myocardial infarction in the rat as well as in the failing human heart.

2. Methods

2.1. Study protocol, myocardial infarction, and hemodynamic measurements

Left coronary artery ligations were performed in adult male Wistar rats (250–300 g) as previously described [9]. Treatment was started 10 days after surgery with placebo, trandolapril (0.3 mg/kg per day), or irbesartan (50 mg/kg per day). Hemodynamic studies were performed 10 weeks after coronary artery ligation under barbiturate anaesthesia and controlled respiration as described [9]. The Standing Committee on Animal Research from our institution has approved the animal study protocol.

2.2. Sample collection, determination of infarct size, and ventricular remodeling

After hemodynamic measurements, hearts were excised and dissected into atria, right, and left ventricle including septum. The left ventricle was cut into three transverse sections: apex, middle ring (3 mm), and base as previously reported [10]. From the middle ring, 5-μm sections were cut at 100-μm intervals and stained with picrosiris red. The boundary lengths of the infarcted and non-infarcted endocardial and epicardial surfaces were traced with a planimeter digital image analyser. Infarct size (fraction of the infarcted left ventricle) was calculated as the average of all slices and expressed as the percentage of length of circumference. Left ventricular cavity area (area enclosed by left ventricular endocardial circumference) was taken as an index of left ventricular dilatation.

2.3. Human subjects

Transmural myocardial tissues from failing human hearts were harvested at the time of transplantation from the left ventricle of patients with dilated cardiomyopathy (DCM, n = 15), or ischemic cardiomyopathy (ICM, n = 8) and rapidly frozen in liquid nitrogen. A total of 12 DCM and six ICM patients received an individual combination of angiotensin converting enzyme (ACE) inhibitor, digoxin, and diuretics. One patient with ICM received an angiotensin antagonist, while three patients with DCM and one with ICM received neither an ACE inhibitor nor an angiotensin antagonist. Normal control hearts were collected from young men at surgery (unused donor hearts) after motor accidents (n = 3). The study was approved by the ethic committee of the University of Würzburg.

2.4. Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic proteins were extracted from human frozen myocardium or from left ventricular myocardium (septum) of rats immediately after the animals had been sacrificed [11]. After Dounce homogenization myocardium was lysed for 10 min on ice in a solution containing 10 mM Hepes (pH 7.6), 10 mM KCl, 1.5 mM MgCl₂, 0.5% Nonidet-40, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were precipitated by centrifugation at 800×g for 30 s, supernatants saved as cytosolic extracts, and the nuclei resuspended in a solution of 20 mM Hepes, 1.5 mM MgCl₂, 420 mM KCl, 0.2 mM EDTA, 1 mM DTT, and 0.5 mM PMSF. The mixture was incubated on ice for 30 min, the supernatant collected after centrifugation for 15 min at 13 000×g, and an equal amount of glycerol buffer added (20 mM Hepes, 100 mM KCl, 0.2 mM EDTA, 20% glycerol).
2.5. Electromobility shift assay (EMSA)

Electromobility shift assays were performed as described [12]. Binding reactions were performed with 10 μg of nuclear protein. Control reaction mixtures contained a 100-fold excess of unlabeled oligonucleotide and were incubated with nuclear extracts as indicated. DNA complexes were separated on a 5% non-denaturing polyacrylamide gel in Tris-borate EDTA buffer. NF-κB oligonucleotides were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

2.6. ELISA NF-κB and AP-1

p65 activity of nuclear extracts as well as AP-1 c-JUN was measured with an ELISA (Active Motif, Rixensart, Belgium) according to the manufacturer’s protocol. OD values were normalized by a provided positive control. All measurements were done in duplicate. The p65 ELISA was validated by comparison with standard gel shifts (n=48).

2.7. ELISA cytokines

rIL-1β (Endogen, Woburn, MA), and rTNF (Endogen, Woburn, MA) were measured in cytosolic extracts, RIPA buffer tissue extracts, and serum according to the manufacturer’s protocol.

2.8. Western blotting

For Western blots myocardial samples were homogenized in ice-cold RIPA buffer (150 mmol/l NaCl, 50 mmol/l Tris–Cl, 5 mmol/l EDTA, 1% v/v Nonidet P-40, 0.5% w/v deoxycholate, 10 mmol/l NaF, 10 mmol/l sodium pyrophosphate, 100 mmol/l phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). Myocardial extracts (20 μg per lane) were mixed with sample loading buffer and separated under reducing conditions on a 10% SDS–polyacrylamide gel. Proteins were electrotransferred onto PVDF membranes (Immun-Blot*, 0.2 μm, Bio-Rad, Munich, Germany) for 90 min at 100 V using a mini trans-blot electrophoretic transfer cell (Bio-Rad, Munich, Germany). Membranes were blocked in blotting solution (20 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.05% Tween 20, pH 7.5) with 5% blocking agent (Amersham, Freiburg, Germany) overnight at 4 °C, followed by incubation with the primary antibody (1:1000, Cell Signaling Technology, Beverly, MA) in blotting solution with 0.5% blocking agent for 2 h at room temperature. The blots were washed five times in blotting solution and incubated with an anti-rabbit IgG hors eradish peroxidase-labeled antibody (1:10 000, Amersham, Freiburg, Germany) for 1 h at room temperature. After extensive washing, bands were detected using the enhanced chemiluminescence assay (ECL+Plus, Amersham, Freiburg, Germany).

2.9. Immunohistochemistry

Cryostat sections of rat and human heart tissue were prepared in the standard manner, fixed with acetone for 10 min and rinsed in PBS with 0.1% Tween-20. Cells were labeled by the sequential application of the primary p65 antibody (1/3200, sc-372, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), an anti-rabbit immunoglobulin and a peroxidase anti-peroxidase complex followed by labeling (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). The slides were washed, dehydrated and mounted for light microscopy.

2.10. Statistical analysis

All replicate data are expressed as mean and standard error of the mean. Absolute differences among groups were compared using a two-way ANOVA adjusted by the Fisher rule. Statistical significance was achieved when two-tailed P<0.05. Statistical analyses were carried out using StatView statistics program (Abacus Concepts, Berkeley, CA).

3. Results

3.1. NF-κB and AP-1 are activated in chronic heart failure

There were no significant differences in infarct size between the groups treated with placebo (48.6±1.6%), the ACE inhibitor trandolapril (52.2±2.1%), or the angiotensin type 1 receptor blocker irbesartan (49.7±2.7%). At 10 weeks after experimental myocardial infarction (MI), NF-κB activity in the non-infarcted myocardium increased ~2.5-fold (Fig. 1). NF-κB activation was negatively correlated with infarct size (r=−0.70, P=0.006; Fig. 2) and left ventricular cavity area (r=−0.85, P=0.002; Fig. 2). AP-1 and its trigger JNK (c-Jun NH2-terminal kinase) were significantly activated in MI when compared with sham operated animals (Fig. 1). The rise in AP-1 activation did not significantly correlate with infarct size (r=0.46, P=0.15).

In human myocardium, NF-κB was about 2-fold increased in patients with heart failure due to dilated cardiomyopathy (control 0.105±0.015, n=3, vs. DCM 0.207±0.017, n=15, P=0.04) or ischemic cardiomyopathy (ICM 0.234±0.035, n=8, P=0.02) (Fig. 3). AP-1 was significantly higher in patients with DCM (control 0.031±0.004, n=3, vs. DCM 0.167±0.012, n=19, P=0.0002) and ICM (0.119±0.014, n=7, P=0.01). There were no statistical differences between patients on ACE inhibitor (n=18), angiotensin antagonist (n=1), or no treatment (n=4). However, the number of patients on angiotensin antagonists or no treatment was rather low.
Fig. 3. NF-κB and AP-1 are activated in human dilated (DCM) or ischemic cardiomyopathy (ICM). Quantitative analysis by ELISAs of NF-κB and AP-1 activation are shown. Explanted left ventricular myocardium was harvested at the time of transplantation from patients with DCM or ICM.

3.2. Immunohistochemistry

Immunohistochemically, activation of NF-κB can be visualized by the translocation of p65 from the cytoplasm to the nucleus. In sham operated animals only minimal nuclear immunoreactivity was observed. In contrast, in infarcted animals and in human failing hearts nuclear translocation of p65 could readily be detected (Fig. 4). In the non-infarcted myocardium, activated NF-κB in nuclei could be mainly localized in cardiac myocytes, and to a lesser extent in endothelial cells and infiltrating leukocytes. In the infarcted myocardium myocytes were replaced by scar tissue with immunoreactivity for nuclear NF-κB in scattered mononuclear cells and fibroblasts.

3.3. Effects of trandolapril and irbesartan treatment on NF-κB and AP-1 activation

Treatment with the angiotensin antagonist irbesartan had no effect on NF-κB or AP-1 activation when compared to placebo treated, infarcted animals. However, treatment with the ACE inhibitor trandolapril led to an additional significant increase in nuclear translocation of NF-κB and AP-1 (Table 1).

3.4. Myocardial IL-1β and TNF expression in chronic myocardial infarction

TNF and IL-1β protein levels were measured from the septal myocardium or in the serum. No statistically significant differences were detected between the groups for TNF and IL-1β in the myocardium (Table 1) or in the serum (data not shown) despite a tendency towards lower IL-1β values in animals treated with an ACE inhibitor (P = 0.08). TNF and IL-1β were not correlated to infarct size. There was no relation between NF-κB activation and TNF expression.
Fig. 4. Immunohistochemical analysis of NF-κB in rat and human cardiac muscle. Photomicrographs (×400) are shown from rat hearts 10 weeks post myocardial infarction and human failing hearts collected at the time of transplantation. Activated p65, visualized by nuclear translocation of p65, was mainly immunolocalized to cardiac myocytes in experimental and human heart failure (representative nuclei are marked).
Several years after the first description of NF-κB by Sen and Baltimore [13], NF-κB has been implicated in numerous important physiologic and pathophysiologic processes including the control of apoptosis, immune functions, and embryonic development [14]. In the heart, a role of NF-κB has almost exclusively been shown in ischemia/reperfusion experiments [15] or in the early phase of myocardial infarction [16]: in a rat in vivo model of ischemia/reperfusion NF-κB increases biphasically with peak levels after 15 min and 3 h [17,18]. Inhibition of NF-κB by introduction of an NF-κB decoy cis element, a synthetic doublestranded DNA with high affinity for NF-κB, in vivo significantly reduced infarct size in ischemia and reperfusion [19]. Furthermore, NF-κB is activated in the late phase of ischemic preconditioning induced by reactive oxygen species, nitric oxide, protein kinase C, and tyrosine kinases [20]. Inhibition of NF-κB by the unspecific inhibitor diethyldithiocarbamate (DDTC) could not only block NF-κB activation, but also abrogate the cardioprotective effects of preconditioning indicating that NF-κB activation is essential for the development of this phenomenon in vivo. Like NF-κB, AP-1 is activated in the early phase of myocardial infarction [16]. However, functional data do not exist. In summary, all these data conclusively demonstrate NF-κB and AP-1 as rapidly induced, central regulators of stress responses in the heart.

Nevertheless, AP-1 and NF-κB are involved in the control of many genes, which not only play a role in acute myocardial injury but also in chronic heart failure. This includes the activation of a number of immune response proteins like inflammatory cytokines (TNF, IL-1β, IL-6 and IL-8) as well as iNOS, and also several important non-immunologic factors like angiotensin and bradykinin. Conclusively, we demonstrate here the chronic activation of NF-κB and AP-1 in experimental and human end-stage heart failure. Our findings substantially extend the recent description of NF-κB activation in myocardial biopsies of patients with heart failure [21] and left ventricular assist devices (LVAD) [22]. NF-κB activation was reduced when cardiac function and remodeling improved due to a LVAD [22]. However, the interpretation of these results was complicated by the fact that a control group of patients without CHF was missing and all patients received steroids, a medication known to reduce NF-κB activity. In addition, we demonstrate that therapy has a major impact on NF-κB activation: long-term ACE inhibition markedly increased nuclear translocation of NF-κB indicating that changes in treatment regimens of CHF may have influenced previously reported results.

In accordance with previous studies [23,24], we did not observe an increase of TNF or IL-1β protein in the myocardium or serum of animals following myocardial infarction despite NF-κB activation and hemodynamic signs of heart failure. Nevertheless, it is important to note that in a variety of other studies TNF in particular was elevated in models of chronic heart failure [25–27]. However, NF-κB activation without cytokine activation does not have to be counterintuitive since recently NF-κB independent regulation of proinflammatory cytokines was demonstrated in a model of hemodynamic pressure overloading [28]: whereas cytokine expression decreased 72 h after aortic banding, NF-κB remained activated. This effect was not secondary to desensitization since the reaction to LPS was preserved. The authors propose that TTP, a protein capable of destabilizing TNF mRNA, might be responsible for modulating the expression of proinflammatory cytokines following cardiac injury independent of NF-κB.

Interestingly, we could demonstrate a reduction of IL-1β expression with drug treatment in accordance with a study from Wei et al. [29]. This attenuation of the rise in cardiac cytokines was previously demonstrated to be independent of hemodynamics [29] and could thereby influence scar remodeling and inflammatory cell invasion [27]. Antioxidative properties and a reduction in the angiotensin II induced endothelin-1 expression may account for this anti-cytokine effect of ACE inhibitors [29].

Yet, what could the impact be of a sustained activation of acute stress response transcription factors in chronic heart failure? First, since most of the immune proteins elevated in heart failure contain NF-κB binding sites in their promoters, sustained activation of NF-κB is a pos-

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>MI+placebo</th>
<th>MI+trandolapril</th>
<th>MI+irbesartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>16</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>NF-κB</td>
<td>0.043±0.014</td>
<td>0.117±0.013*</td>
<td>0.274±0.025**††</td>
<td>0.117±0.019*</td>
</tr>
<tr>
<td>AP-1</td>
<td>0.144±0.015</td>
<td>0.220±0.024*</td>
<td>0.369±0.047**††</td>
<td>0.285±0.046*</td>
</tr>
<tr>
<td>TNF tissue (pg/μg)</td>
<td>3.76±0.69</td>
<td>3.35±0.85</td>
<td>3.68±0.84</td>
<td>4.41±1.14</td>
</tr>
<tr>
<td>IL-1β tissue (pg/μg)</td>
<td>9.21±1.25</td>
<td>9.86±1.55</td>
<td>6.32±1.1</td>
<td>6.71±2.02</td>
</tr>
</tbody>
</table>

*P<0.05 vs. sham. **P<0.0001 vs. sham. †P<0.05 vs. MI placebo. ††P<0.001 vs. MI placebo.
sible explanation for the increased expression of those host defence proteins in heart failure. Second, it is an indicator that the above-mentioned immune proteins are still actively signaling and not completely uncoupled by counter-regulation. Third, chronic NF-κB and AP-1 activation may also have functional significance: both transcription factors are central elements in the regulation of the innate immune system, of wound healing, of apoptosis, and of cell structure, which play a crucial role in ventricular remodeling. Moreover, evidence exists that activation of NF-κB might even be protective: NF-κB is readily activated by oxidative stress. In neonatal rat cardiac myocytes, inhibition of TLR2 (toll like receptor), an activator of NF-κB, was able to inhibit hydrogen peroxide induced NF-κB activation. This reduction in NF-κB activation led to increased cytotoxicity and apoptosis of myocytes [12]. Considering the important role of apoptosis in CHF and the expression of TLR especially in the border zone between viable and damaged myocardium, one might speculate that activation of NF-κB could play a role in the protection of ischemic but viable myocardium in heart failure.

A cardioprotective effect of sustained NF-κB activation is also a potential explanation for two other phenomena reported here. Larger infarct sizes and a higher degree of dilatation were both significantly correlated with less NF-κB activation. Although not directly shown, these data attribute to NF-κB a beneficial effect against progressive LV remodeling and dilatation. These results are also in accordance with a study investigating the expression of iNOS, a gene that is regulated by NF-κB, in CHF [30]. Herein, lower myocardial iNOS RNA expression was detected in patients with lower left ventricular stroke volume. Furthermore, in our study animals treated with an ACE inhibitor, the standard therapy of heart failure, which has been proven to attenuate LV remodeling, exhibited significantly more activation of NF-κB and AP-1. Again, cardioprotective effects of NF-κB and AP-1 may have a major impact on the treatment benefit. Yet, while chronic ACE inhibition was associated with significantly increased NF-κB and AP-1 activation, angiotensin receptor antagonism had no effect. On the other hand, in two large clinical trials ACE inhibition and AT1 antagonism were not statistically different, although the ACE inhibitor tended to be more effective in patients with large MI (Optimal Trial in Myocardial Infarction with the Angiotensin II Antagonist Losartan (OPTIMAAL) [31]) as well as in patients with heart failure (Losartan Heart Failure Survival Study (ELITE II) [32]). Accordingly, using the same heart failure model employed here, only an ACE inhibitor, but not an AT1 receptor antagonist improved cardiac performance [33], further supporting our hypothesis of a protective NF-κB role.

The reason for the difference between angiotensin 1 receptor subtype blockage and ACE inhibition on NF-κB activation remains speculative. It can not be explained by increased signaling through the angiotensin 2 receptor subtype since NF-κB can be readily activated by either angiotensin receptor [34]. However, as ACE inhibitors also prevent the breakdown of bradykinin, a well-known NF-κB activator, sustained bradykinin levels may account for the increased NF-κB activation by ACE inhibition in chronic heart failure. Finally, the effects of ACE inhibition might be highly dependent on timing and stage of heart failure, since NF-κB was down-regulated by ACE inhibitors in the acute phase of myocardial infarction [35].

In summary, we have demonstrated a sustained activation of NF-κB and AP-1 in chronic heart failure. As higher activity of NF-κB and AP-1 was observed after ACE inhibition and was correlated with less LV dilatation, our data support a central, beneficial role of NF-κB and AP-1 activation in progressive LV remodeling in congestive heart failure.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Fr. 1377/4-1, SFB 355, B10) and Ernst und Berta Grimmke Stiftung (T.M.B.). We thank M.G. Berova and Anna Dembny for their technical support.

References

[10] Bauersachs J, Galuppo P, Fraccarollo D, Christ M, Ertl G. Improvement of left ventricular remodeling and function by hydroxy-methylglutaryl coenzyme a reductase inhibition with cerivastatin in


