Opposing roles of Akt and STAT3 in the protection of the maternal heart from peripartum stress

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Received 21 September 2013; revised 10 January 2014; accepted 12 January 2014; online publish-ahead-of-print 20 January 2014

Time for primary review: 31 days

Background

Peripartum cardiomyopathy (PPCM) is a pregnancy-associated cardiomyopathy in previously healthy women. Mice with a cardiomyocyte-restricted deletion of signal transducer and activator of transcription-3 (STAT3, CKO) develop PPCM. PI3K-Akt signalling is thought to promote cardiac hypertrophy and protection during pregnancy. We evaluated the role of activated Akt signalling in the maternal heart postpartum.

Methods and results

CKO mice were bred to mice harbouring an Akt transgene, specifically expressed in cardiomyocytes (CAkttg) generating CKO; CAkttg, CAkttg, CKO, and wild-type sibling mice. CAkttg and CKO;CAkttg female mice developed PPCM with systolic dysfunction. Both genotypes displayed cardiac hypertrophy and lower capillary density, showed increased phosphorylation of p66 Src homology 2 domain containing protein and FoxO3A, and reduced expression of manganese superoxide dismutase as well as increased miR-146a levels [indicative for generation of the anti-angiogenic 16 kDa prolactin (PRL)]. Cardiac inflammation and fibrosis was accelerated in CKO;CAkttg and associated with high postpartum mortality. The PRL blocker, bromocriptine (BR), prevented heart failure and the decrease in capillary density in CKO;CAkttg and CAkttg mice. BR attenuated high mortality, up-regulation of CCL2, and cardiac inflammation as well as fibrosis in CKO;CAkttg. PRL infusion induced cardiac inflammation in CKO;CAkttg independent of pregnancy. In neonatal rat cardiomyocytes, PRL and interferon γ (IFNγ) induced the expression of CCL2 via activation of Akt.

Conclusion

Postpartum Akt activation is detrimental for the peripartum heart as it lowers anti-oxidative defence and in combination with low STAT3 conditions, accelerate cardiac inflammation and fibrosis. PRL and its cleaved 16 kDa form are central for Akt-induced PPCM as indicated by the protection from the disease by PRL blockade.

Keywords

Heart failure • Peripartum cardiomyopathy • Inflammation • STAT3 • Akt • Prolactin

This article is part of the Review Focus on Pregnancy-mediated Heart and Vascular Disease

1. Introduction

Peripartum cardiomyopathy (PPCM) is a life-threatening heart disease in women, characterized by a sudden onset of heart failure in the last month of pregnancy and in the first months after delivery, associated with a high morbidity and mortality.1–4 The major common diagnostic criteria are as follows: (i) a systolic dysfunction with an ejection fraction (EF) <45% and (ii) no pre-existing heart disease except PPCM prior to peripartum heart failure.4 Major co-morbidities are pregnancy-associated hypertensive disorders5,6 and inflammation as indicated by up-regulation of pro-inflammatory serum markers such as sFas/Apo1, C-reactive protein, interferon-γ
(IFNγ), tumour necrosis factor-α (TNFα), and interleukin 6. More recent work suggests that many of these different disease-inducing factors merge in a common downstream mechanism, where enhanced oxidative stress plays a central role, which leads to the activation of the protease, cathepsin D (CD), and subsequent cleavage of prolactin (PRL) into an angiostatic and pro-apoptotic N-terminal 16 kDa PRL fragment (16 kDa PRL). The 16 kDa PRL causes massive endothelial damage and myocardial dysfunction, whereby a large part of the adverse effects of 16 kDa PRL seem to be mediated by microRNA-146a (miR-146a). In recent studies, increased endothelial microparticles and anti-angiogenic sFlt-1 have been reported in experimental models of PPCM and in PPCM patients, suggesting the idea that angiogenic imbalance and endothelial damage play key roles in PPCM. Critical signalling pathways for protection of the maternal heart from peripartum stress involve the signal transducer and activator of transcription-3 (STAT3) and/or peroxisome proliferator-activated receptor gamma, coactivator-1 alpha (PGC-1α). Both factors are involved in defence from oxidative stress by up-regulation of anti-oxidative enzymes, such as manganese superoxide dismutase (MnSOD) and for pro-angiogenic stimuli, by up-regulation of vascular endothelial growth factor (VEGF). Observations from PPCM patients with subsequent pregnancies suggest that patients are protected throughout pregnancy from heart failure and that the disease re-emerges mainly in the peripartum phase. During pregnancy PI3K/Akt signalling is highly activated, in part by increased mechanical stress, but also by high levels of circulating pregnancy hormones such as oestrogens. Both PI3K/Akt signalling promotes physiological hypertrophy and seems, at least in part, to be responsible for adaptation and protection of the maternal heart during pregnancy. After the delivery, mechanical stress and oestrogen levels decrease rapidly and postpartum PI3K/Akt signalling is no longer increased.

We invesigated whether enhanced Akt activation in the postpartum maternal heart may compensate for loss of STAT3 and protect the maternal heart from PPCM. We therefore introduced a cardiomyocyte-specific constitutively active Akt transgene (E40K-Akt) into our CKO PPCM model. In contrast to our expectation, the resulting CKO/CaKtg mice displayed a higher postpartum mortality associated with even more hypertrophy, fibrosis, and inflammation, compared not only with wild type, but also with CKO or CaKtg littermates. However, the CaKtg mice also developed a milder form of PPCM, with significantly reduced cardiac function and compromised cardiac vasculature. Molecular analyses revealed evidence for enhanced oxidative stress in both models at least in part caused by activation of p66 Src in female STAT3-KO mice (nulli pari, NP).

### 2. Methods

Cell culture media were obtained from Biochrome. IFNγ was obtained from Immunotools and inhibitors from Calbiochem. The recombinant 16 kDa PRL was essentially produced as described previously. BR (bromocriptine mesilate) was purchased from Novartis (Ulm, Germany) for in vivo use. All other chemicals were purchased from Sigma.

### 2.1 Animal experiments

The generation of mice with cardiomyocyte-restricted deletion of STAT3 (STAT3fl/fl; STAT3KO; CaKtg mice since bromocriptine (BR) attenuated the 16 kDa form, is critically involved in the PPCM phenotype of both signalling. Furthermore, we found that PRL, full length as the cleaved homology 2 domain containing protein (SHC) and impaired FoxO3A oxidative stress in both models at least in part caused by activation of p66 Src and PRL, could promote an inflammatory type of PPCM.

For chronic administration of PRL, osmotic minipumps (Alzet; PRL 400 IU/kg/day) were implanted in sedated mice and analysed after 14 days of chronic infusion. Adenoviruses, Lacz-Ad, or CD-Ad (3 x 10^8 pfu of each virus) were injected directly into the mouse left ventricle (LV) and analysed after 7 days, as previously described. CKO/Akttg and CaKtg females were treated with BR (4 mg/kg/day, Novartis) in drinking water, 3 days before to 3 weeks after delivery, for two consecutive pregnancies. Echocardiography was performed, as described previously.

All animal studies were in accordance with the German animal protection law and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes. All experiments were approved by the Local Institutional Animal Care and Research Advisory Committee and permitted by the local authority.

### 2.2 Cell culture experiments with cardiomyocytes

Isolation and cultivation of neonatal rat cardiomyocytes (NRCM) were performed, as described previously. Stimulation with IFNγ (50–1000 IU/mL, Immunotools) was carried out for 15 min on cardiomyocytes under serum-free conditions in DMEM/M199 (4:1). The Akt inhibitor LY294002 (10 μM) or the STAT3 inhibitor peptide (10 μM) was applied 1 h prior to stimulation with IFNγ (100 IU/mL) or PRL (0.2 IU/mL). NRCM were stimulated with IFNγ and/or PRL for 15 min or 24 h.

### 2.3 qRT-PCR, miR-qRT-PCR, and western blot

Total RNA isolation, real-time PCR, and expression of mature miR-146a (AppliedBioSystems) were performed as described. Protein expression levels were determined by western blotting, using SDS–PAGE as previously described. More detailed information on the primer sequences and antibodies used are provided in the Supplementary material online, Methods.

### 2.4 Histology, immunostaining, and DNA damage quantification

Detailed description on histological analyses, immunohistochemistry, and DNA damage quantification (AP-sites assay, Abcam) is provided in the Supplementary material online, Methods.

### 2.5 Statistical analyses

Data are presented as mean ± SD. Differences between groups were analysed by Mann–Whitney test, log-rank test, Student’s t-test, or ANOVA, followed by Bonferroni as appropriate. A two-tailed P-value of <0.05 was considered statistically significant.

### 3. Results

#### 3.1 Effect of increased cardiomyocyte-specific expression of Akt in female STAT3-KO mice

The crossing scheme to generate sibling CKO/CaKtg (aMHC–CreERT2; STAT3fl/fl; aMHC–Akttg), CKO (aMHC–CreERT2; STAT3fl/fl), CaKtg (aMHC–Akttg), and WT (STAT3fl/fl) and the corresponding western...
largely comparable with WT. Female mice with cardiac-specific overexpression of a constitutively active Akt transgene (E40K-Akt: CAkttg) displayed concentric hypertrophy with a preserved cardiac function and a slightly reduced heart rate at 3 and 6 months of age compared with WT (Table 1). The cardiac phenotype of CKO;CAkttg was similar to CAkttg with normal cardiac function and morphology in NP CKO female mice (Figure 1A). At 6 months of age, a moderately increased fibrosis was present in CAkttg (two-fold, Figure 2A). Highly morbid CKO:CAkttg mice or CKO:CAkttg mice found dead in the cage showed severe oedemas (body weight up to 60 g) and enlarged and dilated hearts indicative for heart failure.

After two pregnancies surviving CKO, CAkttg and CKO;CAkttg displayed heart failure with the lowest LV function present in CKO:CAkttg compared with WT (Figure 2B). Postpartum CKO, CAkttg, and CKO;CAkttg hearts were enlarged and dilated compared with WT, and heart weight (HW) and HW/body weight (BW) ratio, as well as CSA, were significantly higher in postpartum CAkttg and CKO;CAkttg compared with postpartum WT mice (Figure 2B and C, Table 2). Due to different cardiomyocyte size of the mutant and WT mice, capillary density was determined per LV area. The capillary density was lower and the degree of fibrosis was higher in CKO, CAkttg, and CKO;CAkttg compared with WT hearts (Figure 2D–G). The degree of inflammatory infiltrates, as indicated by positive staining for the pan-inflammatory marker CD45, was higher in CKO and CAkttg hearts compared with WT and highest in CKO;CAkttg mice (Figure 2H and I). Staining for macrophage marker MOMA2 and granulocyte marker Ly-6G + C indicated that numerous macrophages and granulocytes were present in postpartum hearts from CKO:CAkttg mice (Figure 2J).

### 3.2 Postpartum cardiomyopathy in CAkttg and CKO:CAkttg

As reported previously, CKO mice develop PPCM with increased mortality in relation to the number of pregnancies (Figure 2A). Postpartum survival of CAkttg females was comparable with WT, while the postpartum survival rate of CKO:CAkttg was substantially lower compared with WT (Figure 2A). Highly morbid CKO:CAkttg mice or CKO:CAkttg mice found dead in the cage showed severe oedemas (body weight up to 60 g) and enlarged and dilated hearts indicative for heart failure.

### 3.3 Evidence for increased oxidative stress and generation of the anti-angiogenic 16 kDa PRL in postpartum CAkttg and CKO:CAkttg

We previously reported that, in CKO mice with PPCM, the anti-oxidative MnSOD was reduced. Here we observed that cardiac MnSOD mRNA expression is also reduced in postpartum CAkttg (−39 ± 27%, P < 0.05 vs. WT-PP) and in postpartum CKO:CAkttg mice (−62 ± 9%, P < 0.05 vs. WT-PP), suggesting increased oxidative stress in postpartum hearts of both genotypes (Figure 3A). It has been shown that oxidative stress activates p65SHC adaptor protein (SHC) in the cardiovascular system. Moreover, Akt signalling can be modulated by activated SHC and, thereby, selectively phosphorylate with this inactivate FoxO3A, a known transcription factor that regulates MnSOD. We observed increased phosphorylation of SHC and of FoxO3A in postpartum hearts from CKO, CAkttg, and CKO:CAkttg mice compared with WT mice (Figure 3B). Furthermore, we detected an enhanced DNA damage (AP sites) as a marker for oxidative stress in CKO:CAkttg PP compared with WT PP hearts (Figure 3D). CAkttg

### Table 1 Cardiac function, morphometry, and morphology in mice NP 6 months of age

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CKO</th>
<th>CAkttg</th>
<th>CKO;CAkttg</th>
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<tbody>
<tr>
<td>%FS</td>
<td>44 ± 9</td>
<td>34 ± 2**</td>
<td>41 ± 10</td>
<td>37 ± 9</td>
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<td>LVEDD (mm)</td>
<td>3.5 ± 0.2</td>
<td>3.4 ± 0.3</td>
<td>3.8 ± 0.4</td>
<td>3.7 ± 0.2*</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.0 ± 0.4</td>
<td>2.3 ± 0.2*</td>
<td>2.2 ± 0.4</td>
<td>2.4 ± 0.4*</td>
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<td>HR (bpm)</td>
<td>505 ± 52</td>
<td>491 ± 57</td>
<td>475 ± 80</td>
<td>436 ± 35**</td>
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<tr>
<td>HW (mg)</td>
<td>122 ± 20</td>
<td>110 ± 27</td>
<td>146 ± 34</td>
<td>169 ± 32**</td>
</tr>
<tr>
<td>BW (g)</td>
<td>23 ± 1</td>
<td>24 ± 2</td>
<td>24 ± 1</td>
<td>25 ± 1**</td>
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<td>HW/BW</td>
<td>5.2 ± 0.9</td>
<td>4.6 ± 0.8</td>
<td>6.5 ± 1.1*</td>
<td>5.7 ± 1.1**</td>
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<tr>
<td>CSA (μm²)</td>
<td>723 ± 116</td>
<td>683 ± 117</td>
<td>949 ± 188*</td>
<td>948 ± 31***</td>
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</tbody>
</table>

Data are means ± SD. Fractional shortening (FS), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and heart rate were determined by echocardiography and heart rate (beats per minute, bpm) in n = 8 WT, n = 10 CKO, n = 14 CAkttg, n = 12 CKO:CAkttg mice determined at 6 months of age.

**P < 0.05, ***P < 0.01 vs. WT.
hearts tended to have more DNA damage as well (Figure 3D). We showed previously that enhanced oxidative stress in postpartum hearts of CKO mice leads to subsequent activation of CD. While neither NP CAktg nor CKO;CAktg displayed increased expression of the pro-form and the activated form of CD, both genotypes exhibited increased levels of both forms postpartum when compared with WT (Figure 3C, see Supplementary material online, Figure S1A). Increased CD activity promotes cleavage of the nursing hormone PRL into the angiostatic N-terminal 16 kDa PRL form. Beside CD, MMP3 is also able to cleave PRL in endothelial cells. We observed substantially higher levels of the pro-form and the active form of MMP3 (Figure 3E, pro-form: three- to five-fold, active form: two-fold, P < 0.05 vs. postpartum WT) in postpartum CKO;CAktg mice, while postpartum CAktg displayed no such increase (Figure 3E). In addition, we found that PRL moderately induces MMP3 in neonatal rat cardiac fibroblasts (NRCF) and in NRCM, and 16 kDa PRL markedly induces MMP3 expression in NRCF but not in NRCM (see Supplementary material online, Figure S2A–D). We showed that 16 kDa PRL promotes the up-regulation of microRNA-146a in postpartum hearts of CKO mice, a feature that is largely responsible for PPCM in these mice. Increased miR-146a levels were present in LV tissue of postpartum CAktg and CKO;CAktg mice compared with WT mice (Figure 3F).

Monocyte chemoattractant protein-1 (CCL2) is a major chemotactic factor for monocytes and a key factor for initiating the inflammatory process. It is up-regulated by full-length PRL in macrophages and by 16 kDa PRL in endothelial cells. We observed that CCL2 protein levels are substantially higher in hearts of postpartum CKO;CAktg mice compared with all other genotypes (Figure 3G).

Figure 2 Phenotype of postpartum cardiomyopathy in CAktg and CKO;CAktg mice. (A) Kaplan–Meier plot (survival curve) depicting survival after two pregnancies (**P < 0.01 vs. WT by log-rank test; n = 12–23). (B) LV dimensions of in situ fixed whole hearts of WT, CAktg, CKO, and CKO;CAktg mice after two pregnancies; bar = 4.2 mm. (C) Haematoxylin and eosin staining of in situ fixed LV sections after two pregnancies of all genotypes; bar = 4.2 mm. (D) LV cryosections stained with isoelectin B4 (blood vessels, yellow), WGA (cell membranes, red), and nuclei (DAPI, blue), scale bar: 50 μm. (E) capillaries to area (μm²) ratio. (F) Sirius red staining depicts collagen fibres in postpartum hearts, scale bar: 50 μm. (G) Bar graph summarizes the degree of fibrosis in arbitrary units in relation to WT (set 1 arbitrary unit). (H) Immunohistochemical staining with the pan-inflammatory marker CD45 (brown staining; counterstained with eosin, red), scale bar: 50 μm. (I) Bar graph summarizes the degree of CD45-positive infiltrates per area in relation to WT (set 1 arbitrary unit). (J) Macrophages stained with MOMA2 and granulocytes stained with Ly-6G + C, (brown staining; counterstained with eosin, red) and respective IgG controls on LV cryosections of CKO;CAktg postpartum mice, scale bar: 50 μm. (**P < 0.05, ***P < 0.01 vs. WT postpartum (PP), n = 7 WT PP, n = 8 CAktg PP, n = 12 CKO PP, n = 14 CKO;CAktg PP). All data are mean ± SD.
Table 2  Cardiac function, morphometry, and morphology in mice after two pregnancies and nursing period

<table>
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<tr>
<th></th>
<th>WT</th>
<th>CKO</th>
<th>CAkttg</th>
<th>CAkttg BR</th>
<th>CKO;CAkttg</th>
<th>CKO;CAkttg BR</th>
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</thead>
<tbody>
<tr>
<td>%FS</td>
<td>35 ± 9</td>
<td>28 ± 6*</td>
<td>27 ± 7*</td>
<td>45 ± 3§§</td>
<td>19 ± 6**</td>
<td>28 ± 9#</td>
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<tr>
<td>LVEDD (mm)</td>
<td>3.8 ± 0.3</td>
<td>4.1 ± 0.4</td>
<td>4.6 ± 0.6**</td>
<td>4.3 ± 0.2§§</td>
<td>4.8 ± 0.3**</td>
<td>4.3 ± 0.5#</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>2.4 ± 0.5</td>
<td>3.0 ± 0.5</td>
<td>3.4 ± 0.5**</td>
<td>2.3 ± 0.2§§</td>
<td>3.9 ± 0.3**</td>
<td>3.1 ± 0.6**##</td>
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<tr>
<td>HR (bpm)</td>
<td>520 ± 71</td>
<td>483 ± 68</td>
<td>466 ± 79</td>
<td>450 ± 34</td>
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</tr>
<tr>
<td>HW (mg)</td>
<td>118 ± 16</td>
<td>123 ± 14*</td>
<td>216 ± 57**</td>
<td>222 ± 39</td>
<td>192 ± 27§§</td>
<td>166 ± 22*</td>
</tr>
<tr>
<td>BW (g)</td>
<td>26 ± 3</td>
<td>26 ± 3</td>
<td>27 ± 2*</td>
<td>28 ± 2</td>
<td>27 ± 2</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>HW/BW</td>
<td>4.5 ± 0.2</td>
<td>4.8 ± 0.7</td>
<td>7.8 ± 1.7*</td>
<td>8.0 ± 0.1**</td>
<td>7.1 ± 0.7**</td>
<td>5.9 ± 0.6**##</td>
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<tr>
<td>CSA (μm²)</td>
<td>952 ± 94</td>
<td>1073 ± 188</td>
<td>1326 ± 136**</td>
<td>1105 ± 32§§</td>
<td>1794 ± 240**</td>
<td>1071 ± 165##</td>
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Data are means ± SD. Fractional shortening (FS), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), and heart rate were determined by echocardiography and heart rate (beats per minute, bpm) in n = 11 WT, n = 11 CKO, n = 12 CAkttg, n = 5 CAkttg BR, n = 9 CKO;CAkttg, n = 9 CKO;CAkttg BR mice per genotype determined 2 weeks after the second pregnancy.

*P < 0.05, **P < 0.01 vs. WT, §P < 0.05, §§P < 0.01 CAkttg BR vs. CAkttg; #P < 0.05, ##P < 0.01 CKO;CAkttg BR vs. CKO;CAkttg.

Figure 3  Postpartum CAkttg and CKO;CAkttg mice showed evidence for increased oxidative stress and generation of the anti-angiogenic 16 kDa PRL. (A) mRNA level (qRT-PCR) of MnSOD in relation to WT (set 1). (B) P-Ser36-SHC and total SHC, P-FoxO3A, and total FoxO3A and (C) pro- and active CD (Pro-CD, Active-CD) protein levels determined by western blot in LV tissue from NP and postpartum (PP) WT, CKO, CAkttg, and CKO;CAkttg mice. Actin served as loading control. (D) Quantification of DNA damage by AP sites in DNA from WT PP, CAkttg PP, and CKO;CAkttg PP hearts (***P < 0.01 vs. WT PP, n = 4 WT PP, n = 6 CAkttg PP, n = 7 CKO;CAkttg PP). (E) Protein levels of pro-form (P-MMP3) and active form of MMP3 (A-MMP3) in WT NP and PP, CAkttg PP, and CKO;CAkttg PP mice. (F) miR-146a level of LV tissue from WT, CAkttg, and CKO;CAkttg PP mice. (G) CCL2 protein level (western blot) of all genotypes PP and quantification (*P < 0.05, **P < 0.01 vs. WT, n = 9 WT, n = 10 CAkttg, n = 12 CKO, n = 9 CKO;CAkttg) All data are mean ± SD.
3.4 The PRL blocking agent BR attenuates postpartum heart failure and inflammation in CAkt^{tg} and CKO;CAkt^{tg}

We previously reported that blocking PRL by BR prevented onset of PPCM in CKO mice. Similar to the effect in CKO mice, BR provided shortly before delivery and for the first 3 weeks following the delivery, prevented the high postpartum mortality in CKO;CAkt^{tg} mice (Figure 4A). In addition, it attenuated heart failure and LV dilatation in both CAkt^{tg} and CKO;CAkt^{tg} mice (Table 2). BR-treated CKO;CAkt^{tg} mice, but not CAkt^{tg}, displayed lowered HW/BW ratio and reduced CSA in comparison with females not treated (Table 2). Cardiac fibrosis was significantly reduced and capillary density increased in BR-treated CAkt^{tg} and in CKO;CAkt^{tg} mice (Figure 4B and C). BR treatment also reduced the degree of inflammatory infiltrates (Figure 4D), CCL2 mRNA (−37 ± 14%, P < 0.05 vs. not treated CKO;CAkt^{tg}), and protein levels in CKO;CAkt^{tg} mice (Figure 4E). Furthermore, miR-146a level were markedly lower in BR-treated postpartum CKO;CAkt^{tg} mice (Figure 4F).

3.5 PRL promotes inflammation in CKO;CAkt^{tg} mice independent of pregnancy

To evaluate whether PRL per se is able to evoke different inflammatory responses in hearts of WT, CKO, CAkt^{tg}, and CKO;CAkt^{tg} mice, we infused all four genotypes with recombinant sheep PRL (400 iU/kg/day) or saline, using osmotic minipumps for 2 weeks. While control saline infusion had no effect on cardiac inflammation in all four genotypes, PRL infusion increased CD45-positive inflammatory infiltrates (Figure 5A–C) and expression of CCL2 (see Supplementary material online, Figure S3) in CKO;CAkt^{tg} compared with WT, CKO, or CAkt^{tg} mice. Fibrosis, capillary density (see Supplementary material online, Figure S3), and cardiac function [% fractional shortening (FS): with PRL: 37 ± 9 vs. NaCl: 37 ± 5, n.s.] were not different in PRL-treated compared with NaCl-treated CKO;CAkt^{tg} mice. In addition, we found that overexpression of CD, with simultaneous infusion
of recombinant PRL, in WT mice promoted cardiac inflammation, indicating that 16 kDa PRL promotes cardiac inflammation (Figure 5D).

3.6 In cardiomyocytes IFNγ and PRL induce activation of CCL2 via Akt signalling, a feature that is potentiated by the combination of the two factors

We showed previously that elevated markers for inflammation are present in almost all African PPCM patients and that, in these patients, high serum levels of IFNγ, PRL, and oxLDL correlate with an adverse outcome in PPCM. To investigate a mechanistic connection between these three factors for cardiac inflammation, we stimulated NRCM in culture with IFNγ and/or recombinant PRL. As shown in Figure 5E, IFNγ induced activation of Akt, STAT1, and STAT3. PRL induced activation of STAT1, STAT5, and Akt but not of STAT3 in NRCM (Figure 5F). IFNγ and PRL induced the expression of CCL2 mRNA in NRCM, an effect that was potentiated by the combination of the two factors (Figure 5G). Pharmacological inhibition of Akt with LY 294002 reduced CCL2 mRNA expression in NRCM stimulated with IFNγ and PRL (Figure 5G). Pharmacological inhibition of STAT3 with the STAT3 inhibitor peptide had no significant effect on IFNγ-mediated induction of CCL2 expression in NRCM (Figure 5H).

4. Discussion

The maternal heart needs strong protection against stress factors induced by physiological and pathophysiological stimuli during pregnancy, delivery, and the early postpartum phase. It is assumed that the
PI3K/Akt signalling pathway activated by oestrogens and potentially also by increased mechanical stimuli (i.e. increased volume load of the heart during pregnancy) plays an important role in cardioprotection during pregnancy. The decrease in Akt activation in the postpartum maternal heart is likely a consequence of the rapid decline in circulating oestrogens and the unloading of the heart after delivery. In turn, STAT3 and PGC-1α appear to be factors specifically needed in the peripartum phase to protect the maternal heart from enhanced oxidative stress and circulating anti-angiogenic factors by up-regulating anti-oxidative enzymes, such as MnSOD, and pro-angiogenic factors, such as VEGF. Here we evaluated whether enhanced activation of Akt in cardiomyocytes may compensate for loss of STAT3 activation in the peripartum heart, with the idea that activation of this pathway may be an additional therapeutic option in this disease. However, our data clearly show that this is not the case, as Akt activation under reduced STAT3 conditions accelerates peripartum heart failure and mortality as observed in CKO;CAkttg mice. Moreover, even peripartum activation of Akt with intact cardiac STAT3 signalling leads to PPCM with enhanced hypertrophy and reduced cardiac function. Similar to our observations in CKO and in PGC-1α knockout mice, we noted increased activation of p66SHC and reduced expression of MnSOD in postpartum hearts from CKO;CAkttg and CAkttg mice, suggesting enhanced oxidative stress, a feature that was further confirmed by increased DNA damage. In line with reduced anti-oxidative capacity, because of lower MnSOD expression, we found enhanced levels of activated CD, a protein known to be activated in the heart by oxidative stress and which, upon activation, efficiently cleaves PRL into its anti-angiogenic 16 kDa form. We have described previously MMP3 as the MMP whose expression is increased in CKO mice with PPCM. In addition, MMP3 is known to promote dilated cardiomyopathy and is able to activate other MMPs. Finally, MMP3 is able to cleave PRL in its 16 kDa form. We observed that the active and inactive forms of MMP3 are substantially up-regulated in CKO;CAkttg mice, but not WT or CAkttg mice. Full-length PRL and/or 16 kDa PRL are inducing MMP3 expression in cardiac fibroblasts and in cardiomyocytes suggesting a positive feedback loop for MMP3 expression and generation of 16 kDa PRL in CKO;CAkttg, which may contribute to the more severe form of PPCM in this genotype compared with CAkttg. We also showed that 16 kDa PRL induces the expression of miR-146a, which mediates most of the adverse effects of 16 kDa PRL in PPCM. The findings that miR-146a levels were increased in postpartum hearts from CKO;CAkttg and CAkttg mice and the fact that BR treatment attenuated PPCM in both genotypes supports the idea that enhanced oxidative stress, PRL cleavage, and the 16 kDa PRL are disease causing in these two novel mouse models of PPCM (Figure 6). Thus, it seems that the deregulation of multiple upstream signalling pathways, i.e. STAT3 or PGC-1α and/or Akt merge in a common pathway where the generation of 16 kDa PRL appears to be the common link (Figure 6).

With regard to the anti-oxidative capacity, we showed that MnSOD is crucial for cardioprotection from enhanced peripartum oxidative stress, as mice hemizygote for MnSOD developed PPCM, MnSOD is reduced in postpartum CKO mice, and treatment of CKO mice with the MnSOD mimeticum MnTBAP protected partially them from PPCM. Here we found that MnSOD expression was reduced in hearts from CAkttg mice, suggesting that activation of Akt in the peripartum heart is suppressing the expression of this gene. The combination of both STAT3 deficiency in CKO;CAkttg mice did not further accelerate the decrease of MnSOD expression, suggesting that both pathways may merge in the regulation of a common transcription factor. In this regard, it is known that the transcription factor FoxO3A regulates the expression of MnSOD. FoxO3A becomes active by de-phosphorylation and translocates to the nucleus, a feature that is promoted by activated STAT3. In turn, Akt signalling modulated by the p66SHC adaptor protein (SHC) can selectively phosphorylate and, with this, inactivate FoxO3A, which subsequently leads to a down-regulation of MnSOD. This specific Akt signalling pathway does not regulate GSK3. Here we observed that SHC and FoxO3A phosphorylation were higher in postpartum hearts form CKO, CAkttg, and CKO;CAkttg mice compared with WT. In addition, activation of p66SHC is of even broader importance for the regulation of the intracellular redox balance as in addition to its negative regulation of MnSOD, it triggers NADPH membrane oxidase-induced ROS production and it migrates to the mitochondrial intermembrane space where it promotes the production of H₂O₂. These data suggest that STAT3 and Akt play opposing roles in the postpartum heart in accordance with a circuit involving p66SHC and FoxO3A, subsequent regulation of MnSOD, and protection from oxidative stress (Figure 6).

We observed a substantially higher increase in the HW/BW ratio and in CSA, together with a rarification of capillaries in postpartum CAkttg mice, a feature that was even more pronounced in CKO;CAkttg mice. This feature was paralleled by a decrease in cardiac function which was more severe in CKO;CAkttg mice. BR treatment prevented cardiac dysfunction and increased the cardiac capillary density without substantially affecting the degree of hypertrophy in CAkttg mice. These data suggest that the pregnancy-induced cardiac hypertrophy is more pronounced and potentially less reversible in CAkttg, compared with WT mice. The observation that BR treatment is not able to reverse peripartum hypertrophy in the CAkttg mice suggests that this effect is independent of PRL. In turn, the positive effect of BR on peripartum CAkttg, which is associated with increased capillary density, suggests that diminished vasculature most likely caused by the 16 kDa PRL form is causal for PPCM in this genotype.

BR treatment prevented cardiac dysfunction and the decrease in the cardiac vasculature in CKO;CAkttg, suggesting that 16 kDa PRL-mediated pathophysiological effects are also involved in PPCM of this genotype. However, BR treatment also attenuated the massive cardiac hypertrophy and inflammation in the postpartum CKO;CAkttg hearts. Cardiac inflammation can induce cardiac oedema and cardiomyocyte swelling which, subsequently, impairs cardiac function. Treatment with BR attenuated cardiac inflammation and may, therefore, also prevent oedema and cardiomyocyte swelling, a notion that could explain why the increase in HW/BW and CSA was blunted in BR-treated CKO;CAkttg mice. The combination of low STAT3 and high Akt conditions promotes cardiac inflammation in the postpartum heart, which was associated with an increased expression of CCL2, a chemokine known to attract inflammatory cells, mainly macrophages. It was not possible for us to distinguish, in the postpartum CKO;CAkttg hearts, whether CCL2 has primarily been secreted by the stressed cardiomyocytes or whether it is released by the invading inflammatory cells. However, it is likely to be connected with the high levels of circulating PRL in the postpartum phase, since we showed that infusion of PRL alone was able to increase inflammatory infiltrates in CKO;CAkttg hearts, compared with the other three genotypes. In addition, we also showed that the generation of 16 kDa PRL by overexpression of activated CD and infusion of PRL in the heart of WT mice was sufficient to induce cardiac inflammation. While we could not convincingly show that 16 kDa PRL induces CCL2 in cardiomyocytes, it has been shown previously that 16 kDa PRL induces CCL2 expression in endothelial cells, a feature that may...
also contribute to cardiac inflammation in postpartum CKO;CAkttg mice. In an African PPCM collective we have previously observed that higher PRL levels and oxLDL (a marker for oxidative stress) correlated with the failure to cease inflammation, a feature, which predicted poor outcome.\(^7\) In cultured cardiomyocytes, we found that full-length PRL induces Akt, STAT1, and STAT5 activation while STAT3 was not significantly induced. Moreover, PRL induced CCL2 expression in cardiomyocytes, which could be prevented by pharmacological inhibition of Akt. A third factor that correlated with inflammation and poor outcome in this African collective was IFN\(_\gamma\).\(^7\) We observed that IFN\(_\gamma\) induced STAT1, STAT3, and Akt activation and increased the expression of CCL2, a feature that was previously shown by others.\(^31\) The IFN\(_\gamma\)-mediated increase in CCL2 expression could completely be blocked by pharmacological inhibition of Akt, while blocking STAT3 had no effect.\(^31\) The precise mechanism needs to be clarified in future experiments.

In conclusion, our data suggest that enhanced Akt signalling in postpartum hearts is not protective and, in fact, is disease promoting. Furthermore, the present study indicates that deregulation of multiple up-stream factors such as STAT3 or PGC-1\(\alpha\)\(^8,9\) or, as shown here, Akt, in the postpartum heart merge in a common pathway involving a shift in the redox balance towards increased oxidative stress and subsequent generation of 16 kDa PRL that impairs the cardiac vasculature, which finally is leading to PPCM (Figure 6). In addition, postpartum Akt activation in situations predisposed to develop PPCM due to lower STAT3 activation appears to be even more detrimental since it promotes cardiac inflammation, as observed in postpartum CKO;CAkttg mice. Finally, this study is in line with previous experimental data\(^8,9,11\) showing that PRL blockade with BR is able to prevent PPCM in female mice at risk for the disease. A high risk to develop PPCM is also present in PPCM patients with a subsequent pregnancy.\(^13\) Data from a small collective of PPCM patients with subsequent pregnancies suggests that BR may prevent the relapse of the disease.\(^8\) In addition, the beneficial effect of BR in meanwhile four different experimental models of PPCM, CKO,\(^8\) PGC-1\(\alpha\) knockout,\(^9\) CAkttg, and CKO;CAkttg supports the notion that, albeit different aetiologies for the disease are likely, eliminating the common culprit factor PRL, full-length and 16 kDa PRL, seems to be efficient in a broad range of PPCM forms. In line with this idea are a pilot study, case reports and data from the German PPCM registry where BR treatment is, in general, associated with a better recovery and outcome in a highly heterogeneous collective of PPCM patients.\(^5,8,10,32\) However, it is necessary to test the BR therapy concept in larger controlled randomized multicentre trials as the one that we are currently performing in Germany (randomization of 60 PPCM patients to BR-therapy or no BR-therapy, study registered at ClinicalTrials.gov, study number: NCT00998556).
Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank Birgit Brandt, Sergei Erschow, Lea Greune, and Silvia Gutzke for technical assistance, and Thomas Schepeler and Yangxi Zhao (Technical Chemistry, Leibniz University, Hannover) for technical support and fruitful discussions.

Conflict of interest: none declared.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft grant HI 842/4-1 and REBIRTH II.

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