

# Angiotensin II downregulates the fatty acid oxidation pathway in adult rat cardiomyocytes via release of tumour necrosis factor- $\alpha$

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## KEYWORDS

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**Aims** Advanced heart failure is often associated with reduced myocardial fatty acid oxidation capacity. We have previously observed that failing hearts of mice with overexpression of angiotensinogen in the myocardium exhibit marked reduction of key regulatory proteins of fatty acid oxidation. In the present study, we determined whether exposure of adult rat cardiac (ARC) myocytes to angiotensin II (Ang II) influences expression of fatty acid translocase, muscle-type carnitine palmitoyl transferase-I, and medium-chain acyl-CoA dehydrogenase.

**Methods and results** Ang II reduced mRNA expression of the three regulatory proteins in ARC myocytes during the entire 14-days culture period. However, protein expression and palmitate oxidation rate remained unaltered for 7 days, but subsequently markedly decreased. The decrease of protein expression and of fatty acid oxidation coincided with the onset of increased protein expression of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ). The effect of Ang II was completely abolished by either blocking TNF- $\alpha$  formation through inhibition of reactive oxygen species-mediated activation of nuclear factor- $\kappa$ B or by neutralizing TNF- $\alpha$  with a specific antibody. Activation of peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ) and PPAR $\beta/\delta$  counteracted Ang II-mediated reduction of the fatty acid oxidation pathway.

**Conclusion** Prolonged exposure of cardiac myocytes to Ang II elicits downregulation of the fatty acid oxidation pathway mediated by enhanced synthesis of TNF- $\alpha$ .

## 1. Introduction

Cardiac myocytes exposed to chronic stress undergo a sequence of phenotypic changes, which eventually lead to heart failure. A number of observations indicate that adverse ventricular remodelling in response to disease conditions including myocardial infarction,<sup>1,2</sup> pressure<sup>3,4</sup> or volume overload,<sup>5</sup> and tachycardia<sup>6</sup> is associated with changes in myocardial energy metabolism.<sup>4–8</sup> There is increasing evidence that fatty acid oxidation is reduced in advanced stages of ventricular remodelling, and more glucose is oxidized.<sup>7,9</sup> The consequences of reduction of the fatty acid oxidation pathway for disease progression remain controversial. On one hand, the shift to glucose oxidation may protect the myocardium by factors including enhancement of ATP yield per O<sub>2</sub> consumed,<sup>10</sup> support of ion pumps by glycolysis-derived ATP,<sup>11</sup> and reduction of proton production by oxidation, instead of conversion to lactate, of glycolytically produced pyruvate.<sup>12</sup> On the other hand, a

reduction of fatty acid oxidation may limit overall energy production and favour accumulation of potentially toxic lipid intermediates in the cytoplasm ('lipotoxicity').<sup>13</sup>

Reduction of fatty acid oxidation during ventricular remodelling and heart failure seems to be mediated by reduction of protein levels of key regulatory proteins of fatty acid metabolism.<sup>1,6,7,14–16</sup> This has been largely attributed to inactivation and/or downregulation of the nuclear transcription factors peroxisome proliferator-activated receptors (PPARs)  $\alpha$ <sup>17,18</sup> and  $\beta/\delta$ <sup>19</sup> or of the PPAR $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ),<sup>20</sup> which regulate the expression of multiple genes controlling both fatty acid uptake and oxidation. However, a number of observations indicate that, in addition to reduced transcription, levels of regulatory proteins are also modulated by posttranscriptional mechanisms. Specifically, in rodent models of pathological left ventricular remodelling, mRNA expression of enzymes of fatty acid oxidation was reduced during compensated hypertrophy, but protein expression and fatty acid oxidation rate were maintained at control levels. However, both protein expression and fatty acid oxidation dropped dramatically when heart failure occurred.<sup>1,9,14</sup> The signalling pathways driving

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downregulation of the fatty acid oxidation pathway during ventricular remodelling are largely unknown.

Activation of the renin–angiotensin system and increased formation of the main effector angiotensin II (Ang II) play a critical role in left ventricular remodelling and heart failure.<sup>21</sup> Ang II has been implicated in myocyte hypertrophy, interstitial fibrosis, and apoptosis.<sup>22</sup> Furthermore, Ang II promotes the inflammatory response, which may be involved in the progression from compensated remodelling to heart failure.<sup>23</sup> However, at present, little is known about whether Ang II contributes to altered metabolic regulation in the myocardium undergoing remodelling. We have recently observed that mice with targeted overexpression of angiotensinogen in the myocardium, resulting in chronically elevated myocardial Ang II at unaltered blood pressure,<sup>24</sup> progressively develop left ventricular hypertrophy associated with downregulation of mRNA expression of a number of regulatory proteins of fatty acid oxidation.<sup>9</sup> Protein expression and palmitate oxidation, measured during *in vitro* perfusion, did not differ from wild-type mice during compensated hypertrophy, but were significantly reduced after the onset of heart failure. Accordingly, chronic stimulation of the myocardium with Ang II elicits progressive myocardial remodelling, which results in reduction of regulatory proteins of the fatty acid oxidation pathway when heart failure occurs. However, it is not known whether Ang II stimulation of cardiac myocytes directly affects expression of the fatty acid oxidation pathway.

We therefore studied the effects of prolonged stimulation of adult rat cardiac (ARC) myocytes by Ang II on the expression of regulatory proteins of fatty acid oxidation. The results indicate that Ang II elicits downregulation of the fatty acid oxidation pathway. This effect is largely mediated by Ang II-induced generation of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ).

## 2. Materials and methods

An expanded Materials and methods section is available in the online data supplement.

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and was approved by the local animal protection authorities.

### 2.1 Cell isolation and culture

ARC myocytes were isolated from male OFA rats (Charles River, France) as previously described.<sup>25,26</sup> The culture medium was M199 supplemented with 20% (vol/vol) foetal calf serum (FCS; Invitrogen) throughout the culture period. The culture medium contained glucose (5.5 mM), Ang II (1–1000 nM), TNF- $\alpha$  (10 ng/mL), N-acetyl cysteine (NAC; 10 mM), apocynin (1 mM), SN50 (18  $\mu$ M), anti-rat TNF- $\alpha$  antibody (0.2  $\mu$ g/mL), WY-14643 (100 nM), L165-041 (1  $\mu$ M), or ciglitazone (10 nM) were added to the medium during the entire culture period. In selected experiments, palmitate plus oleate (0.05–0.25 mM) at equimolar concentrations complexed to bovine serum albumin (0.2 mM) were added.

### 2.2 Immunofluorescence and cell surface area determination

Cells were fixed with paraformaldehyde and stained ( $\alpha$ -actinin, F-actin, and DNA). Relative cell surface area was calculated from digitized images.

### 2.3 [<sup>3</sup>H]-phenylalanine incorporation

To obtain an index for the rate of *de novo* protein synthesis, ARC were incubated with L-[2,3,4,5-<sup>3</sup>H]-phenylalanine. [<sup>3</sup>H]-phenylalanine incorporation was measured, and normalized to DNA content.

### 2.4 Measurement of intracellular reactive oxygen species

Reactive oxygen species (ROS) were measured by the dichlorofluorescein fluorescence method. Upon reaction with ROS, DCF becomes highly fluorescent and fluorescence was then recorded.

### 2.5 Protein and RNA expression

Western blot and quantitative reverse transcriptase–polymerase chain reaction were performed as in previous studies.<sup>26</sup>

### 2.6 Palmitate oxidation

Palmitate oxidation was estimated based on the release of <sup>14</sup>CO<sub>2</sub> from [1-<sup>14</sup>C]-palmitate.<sup>26</sup>

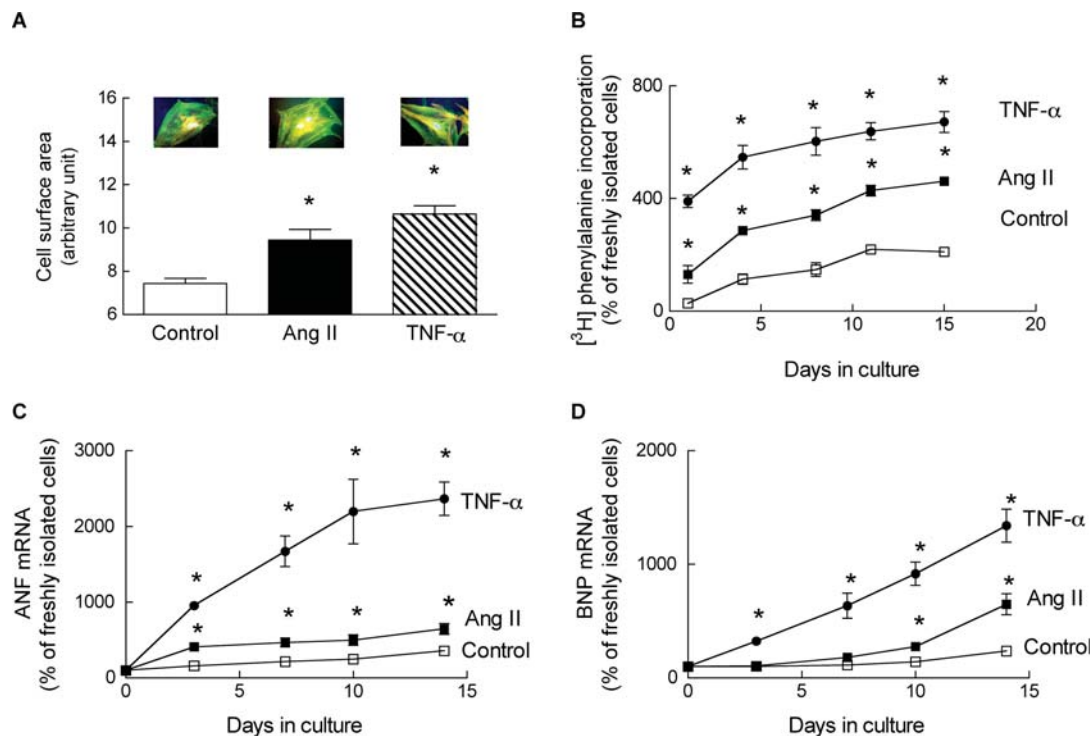
ARC were incubated in sealed flasks containing a suspended filter paper and medium containing palmitate (0.05 mM), oleate (0.05 mM), and 1  $\mu$ Ci/mL [1-<sup>14</sup>C]-palmitate complexed to bovine serum albumin (0.2 mM). <sup>14</sup>CO<sub>2</sub> produced by [1-<sup>14</sup>C]-palmitate metabolism was collected overnight on the filter paper and quantified.

## 3. Results

### 3.1 Ang II and tumour necrosis factor- $\alpha$ elicit reduction of regulatory proteins of fatty acid oxidation in adult rat cardiac myocytes

ARC cultured with 20% FCS underwent phenotypic changes during the 14 days culture period as described previously.<sup>26</sup> Three to 4 days after isolation ARC resumed beating, gradually increased in size, and reexpressed atrial natriuretic factor (ANF) mRNA. Supplementation of the culture medium with Ang II (100 nM) significantly enhanced the hypertrophic response as evidenced by a further increase of cell size, stimulation of protein synthesis, and increase of mRNA expression of both ANF and brain natriuretic peptide (BNP) (*Figure 1*). Since Ang II stimulates synthesis of TNF- $\alpha$ ,<sup>27,28</sup> we exposed, in parallel experiments, cells to TNF- $\alpha$  (10 ng/mL). TNF- $\alpha$  also increased all indices of the hypertrophic response, even more pronounced in extent compared with Ang II (*Figure 1*).

To determine the effects of both Ang II and TNF- $\alpha$  on key regulatory proteins of the fatty acid oxidation pathway, we monitored expression of fatty acid translocase (FAT/CD36), muscle-type carnitine palmitoyl transferase-I (mCPT-I) and medium-chain acyl-CoA dehydrogenase (MCAD). Protein expression of all three regulatory proteins remained stable in control ARC during the 14 days culture period. Both Ang II and TNF- $\alpha$  elicited a gradual reduction of the three regulatory proteins (*Figure 2A–C*). However, there was a clear difference in the time-course. Supplementation of the medium with Ang II during the entire culture period did not affect protein expression up to day 7. Thereafter, protein expression decreased to reach 60% of control on day 14. The reduction of protein expression by Ang II was concentration-dependent (Supplementary material online, *Figure S2*). In contrast to Ang II, TNF- $\alpha$  more rapidly reduced protein expression of FAT/CD36, mCPT-I, and MCAD, starting from the onset of the culture period, to



**Figure 1** Ang II and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) increase indices of myocyte hypertrophy in adult rat cardiac (ARC). (A) Relative difference of ARC size measured after 14 days of culture in control medium or medium supplemented with either Ang II (100 nM) or TNF- $\alpha$  (10 ng/mL). Cells were stained for  $\alpha$ -actinin (green), F-actin (red) and DNA (blue). At least 100 cells in different cultures were measured for each condition. (B) *De novo* protein synthesis, estimated from [<sup>3</sup>H]-phenylalanine incorporation. (C and D) mRNA expression of ANF (C) or brain natriuretic peptide (D).  $n = 4-8$ . Values are means  $\pm$  SEM. \* $P < 0.05$  compared with control.

levels as low as 10% of control after 14 days (Figure 2A-C). Correspondingly, exposure of ARC to TNF- $\alpha$  for 7 days reduced palmitate oxidation (to 30%), whereas Ang II had no effect (Figure 2G). During exposure for more than 7 days both TNF- $\alpha$  and Ang II reduced palmitate oxidation (to 15% and 41%, respectively, after 14 days).

In contrast to protein expression, mRNA expression of FAT/CD36, mCPT-1, and MCAD rapidly decreased after isolation in untreated control ARC to 40 to 60% of values measured in freshly isolated cells (Figure 2D-F). Both Ang II and TNF- $\alpha$  elicited a further reduction of mRNA expression of all three regulatory proteins compared with untreated ARC. We have previously observed that the reduction of mRNA expression in control experiments is related to the low fatty acid concentration in the standard culture medium since it is prevented by the addition of fatty acid to the culture medium.<sup>26</sup>

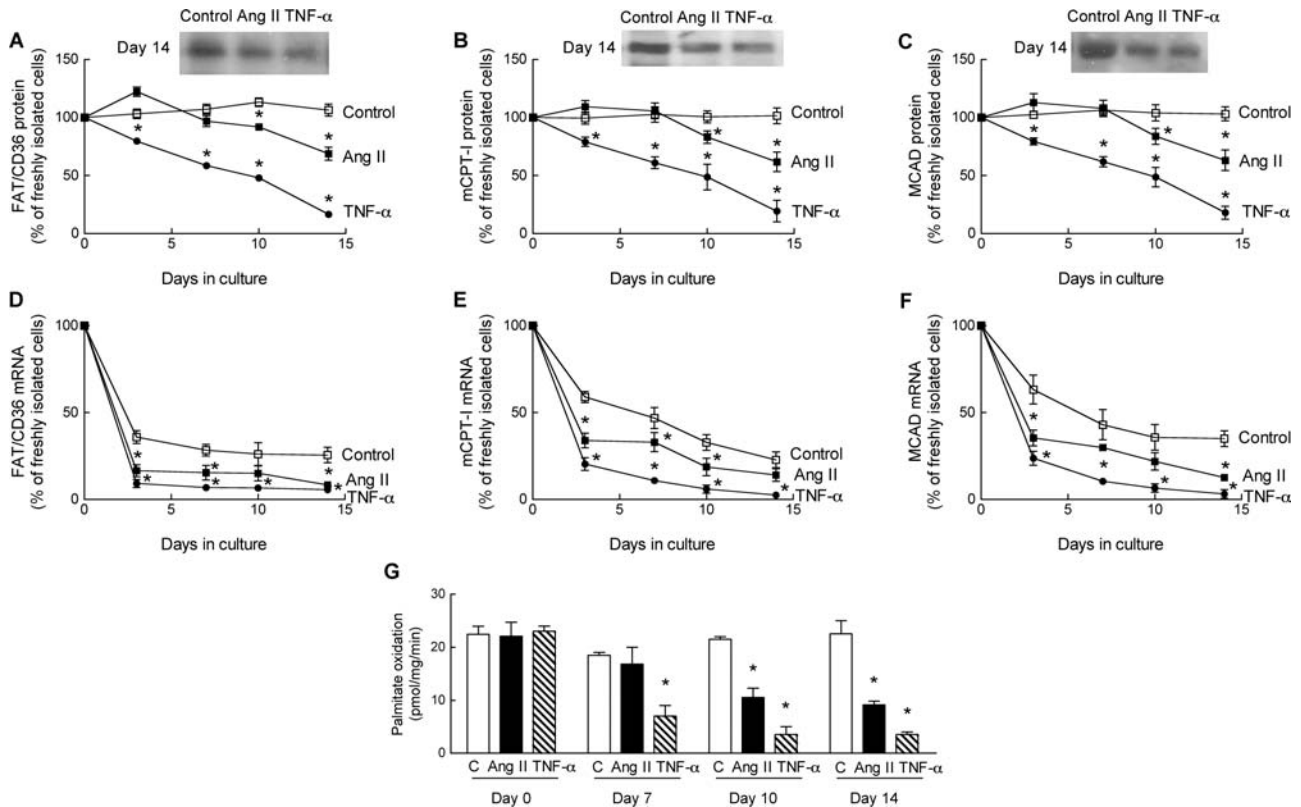
To determine the role of the high concentration of FCS in the effects of Ang II, in additional experiments, ARC cultured in medium containing a low concentration of FCS (1%) were exposed to Ang II (100 nM) or TNF- $\alpha$  (10 ng/mL) for up to 7 days. Both Ang II and TNF- $\alpha$  reduced regulatory proteins of fatty acid oxidation and palmitate oxidation to a similar extent compared with results obtained in the presence of 20% FCS. However, Ang II-induced reduction of both protein expression and palmitate oxidation was detectable earlier, already on day 4, and was reduced on day 7 to values comparable to those observed in the presence of 20% FCS on day 14. The decrease of both regulatory proteins and palmitate oxidation by TNF- $\alpha$  was also accelerated

compared to experiments in ARC cultured with 20% FCS (Supplementary material online, Figure S3).

### 3.2 Ang II-induced reduction of the fatty acid oxidation pathway involves ROS-dependent activation of NF- $\kappa$ B

Because nuclear factor- $\kappa$ B (NF- $\kappa$ B) plays a central role in the Ang II-mediated hypertrophic response,<sup>29</sup> we first measured phosphorylation of the p65 subunit of NF- $\kappa$ B at ser536, which is a marker of NF- $\kappa$ B activation.<sup>30</sup> Total NF- $\kappa$ B protein level was not altered by Ang II (data not shown). However, phosphorylation of NF- $\kappa$ B progressively and concentration-dependently increased in the presence of Ang II until day 10 and then levelled off or slightly decreased (Figure 3A). Ang II at a concentration of 100 nM elicited a maximal response. The Ang II-induced increase of phospho-NF- $\kappa$ B was completely abolished if accumulation of ROS was prevented either by the NADPH oxidase inhibitor apocynin or the ROS scavenger NAC (Figure 3B). Prevention of ROS accumulation was validated in separate experiments by monitoring of dichlorofluorescein diacetate fluorescence (data not shown). Of note, TNF- $\alpha$ , in the absence of Ang II, rapidly and markedly enhanced phosphorylation of NF- $\kappa$ B (Figure 3A).

Inhibition of Ang II-induced ROS activity by apocynin or NAC completely prevented the reduction of protein expression of FAT/CD36, mCPT-1, and MCAD (Figure 3D), and the decrease of palmitate oxidation (Figure 3E). Reduction of both expression of regulatory proteins and palmitate oxidation rate was also prevented by SN50 (Figure 3D and E) which inhibits translocation of NF- $\kappa$ B to the nucleus,



**Figure 2** Both Ang II and tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) reduce expression of key regulatory proteins of fatty acid oxidation, but with different time-course. (A–C) Time-course of protein expression of FAT/CD36 (A), mCPT-I (B), and MCAD (C) in control adult rat cardiac (ARC) (open squares), ARC exposed to Ang II (filled squares), or ARC exposed to TNF- $\alpha$  (filled circles) during the 14-day culture period. (D–F) mRNA expression of FAT/CD36 (D), mCPT-I (E), and MCAD (F). Values in (A) to (F) are expressed as percentage of values measured in freshly isolated ARC. (G) Oxidation of [ $^{14}$ C]-palmitate measured after different culture intervals in control ARC (open bars), ARC exposed to Ang II (black bars), or ARC exposed to TNF- $\alpha$  (hatched bars).  $n$ , at least three determinations for each culture interval and each condition. Values are means  $\pm$  SEM. \* $P < 0.05$  compared with control.

without affecting phosphorylation (Figure 3B). Thus, ROS-mediated activation and translocation of NF- $\kappa$ B is necessary for Ang II-mediated downregulation of the fatty acid oxidation pathway.

### 3.3 Production of tumour necrosis factor- $\alpha$ mediates Ang II-induced reduction of the fatty acid oxidation pathway

To determine the role of TNF- $\alpha$  in Ang II-induced downregulation of regulatory proteins of fatty acid oxidation, we first measured the effect of Ang II on TNF- $\alpha$  expression in ARC. Ang II concentration-dependently stimulated protein expression of TNF- $\alpha$  (Figure 3F). However, TNF- $\alpha$  expression was absent or low during the first 7 days at Ang II concentrations up to 100 nM. Induction of TNF- $\alpha$  protein expression was completely abolished by NAC, apocynin, and SN50 indicating the involvement of the ROS-NF- $\kappa$ B cascade in the stimulation of TNF- $\alpha$  expression (Figure 3G).

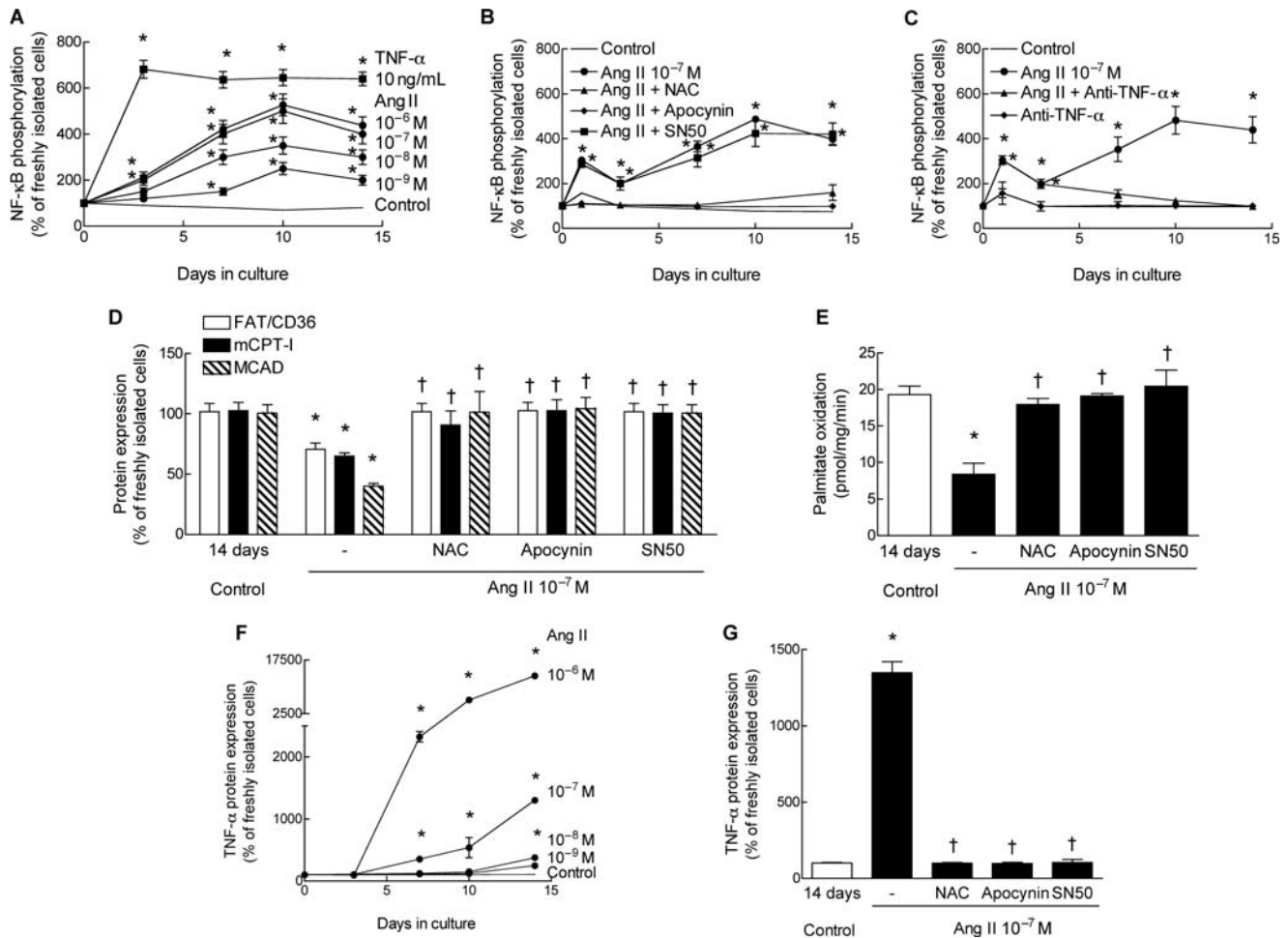
To further evaluate whether TNF- $\alpha$  secreted by ARC in response to Ang II stimulation was sufficient to reduce the fatty acid oxidation pathway, we added an antigen-affinity purified polyclonal rabbit anti-rat TNF- $\alpha$  antibody (0.2  $\mu$ g/mL) to the culture medium. Anti-TNF- $\alpha$  had no effect in unstimulated control cells (data not shown), but completely prevented the Ang II-induced reduction of regulatory proteins of fatty acid oxidation (Figure 4A) and of palmitate

oxidation rate (Figure 4B). Interestingly, anti-TNF- $\alpha$  markedly reduced Ang II-induced phosphorylation of NF- $\kappa$ B in ARC later than 7 days after isolation, suggesting that the generation of TNF- $\alpha$  largely mediated phosphorylation of NF- $\kappa$ B during the second week of culture (Figure 3C). Collectively, these data indicate that downregulation of the fatty acid oxidation pathway in ARC exposed to Ang II is mediated by TNF- $\alpha$ .

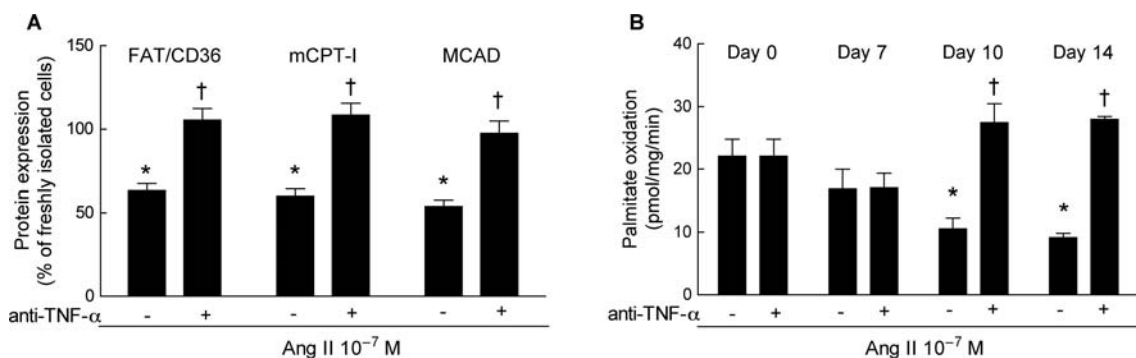
### 3.4 Ang II reduces protein expression of PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ via tumour necrosis factor- $\alpha$

Because several previous studies have implicated reduction of transcriptional activity of PPAR $\alpha$ <sup>17,18</sup> and PPAR $\beta/\delta$ <sup>19</sup> in the downregulation of the fatty acid oxidation pathway during myocardial hypertrophy and heart failure, we next evaluated the effect of Ang II on the expression of three PPAR isoforms PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$ . In control ARC, protein expression of PPARs did not appreciably change during the 14 days culture period (Figures 5A–C).

Ang II elicited a modest, dose-dependent, reduction of protein expression of PPAR $\alpha$ , PPAR $\beta/\delta$ , and PPAR $\gamma$  (Figures 5A–C; Supplementary material online, Figure S2) on day 14. Interestingly, during the first 5 to 7 days of culture expression of PPAR proteins was even slightly higher in ARC exposed to Ang II than in control ARC. In



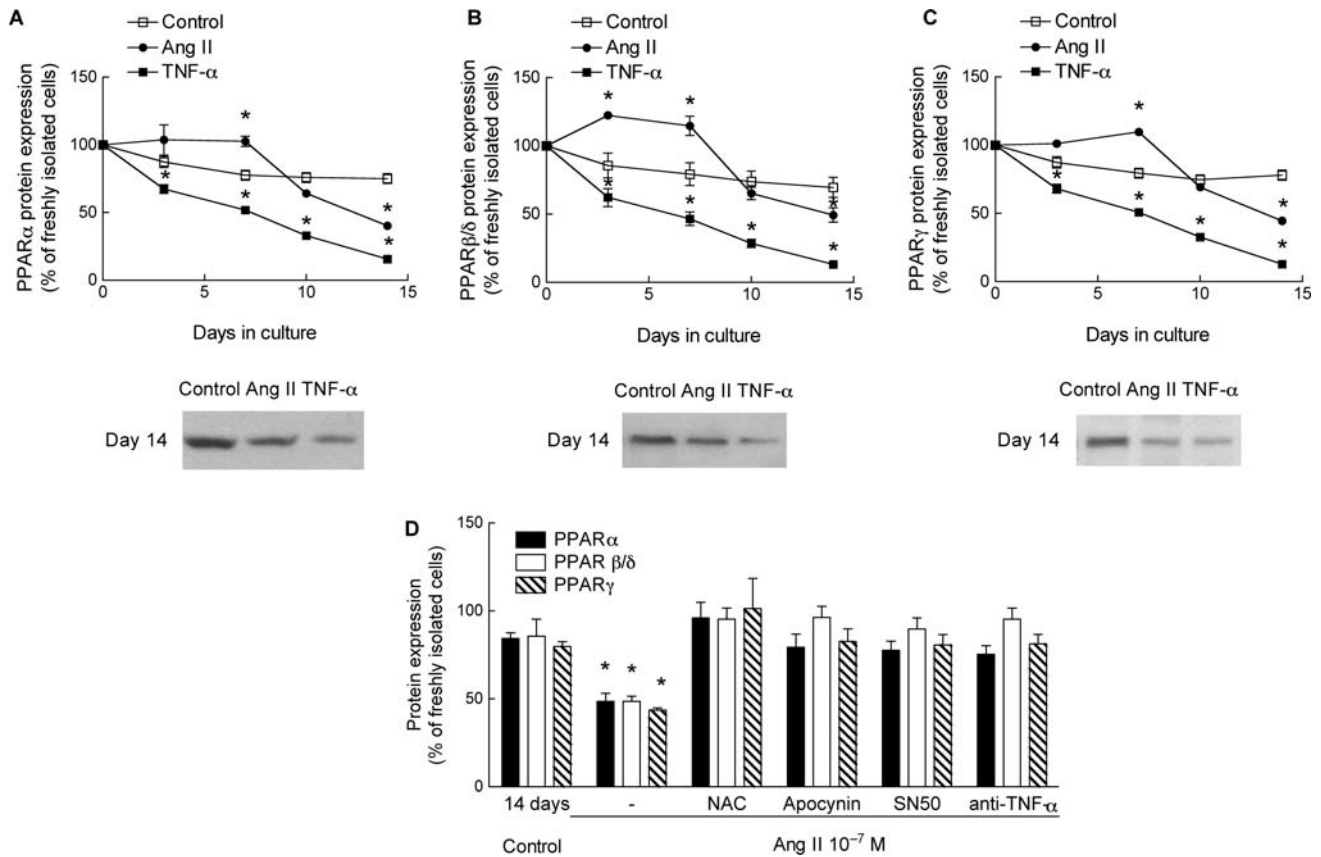
**Figure 3** Ang II elicits phosphorylation of NF-κB, downregulation of the fatty acid oxidation pathway, and enhancement of expression of TNF-α in a ROS-dependent manner. (A) Time-course of Ang II- and TNF-α-mediated phosphorylation of the p65 subunit of NF-κB at ser536. (B) Inhibition of ROS accumulation by either NAC or apocynin-blocked phosphorylation of NF-κB. (C) Binding of TNF-α by an antibody reduced NF-κB phosphorylation during the second week of culture. (D) Inhibition of ROS accumulation by either NAC or apocynin, or inhibition of translocation of NF-κB by SN50 prevented Ang II-mediated reduction of protein expression of FAT/CD36 (open bars), mCPT-I (filled bars), and MCAD (hatched bars). (E) The same interventions also prevented reduction of palmitate oxidation (shown are measurements on day 14). (F) Ang II dose-and time-dependently stimulated protein expression of TNF-α in ARC. (G) Inhibition of ROS accumulation or blockade of NF-κB translocation by SN50 abolished Ang II-mediated synthesis of TNF-α. *n*, at least three determinations for each culture interval and each condition (F and G). All values are means ± SEM. \**P* < 0.05 compared with control (14 days), †*P* < 0.05 compared with Ang II alone.



**Figure 4** Neutralization of TNF-α by an antibody prevents downregulation of regulatory proteins of fatty acid oxidation. (A) Ang II-induced reduction of protein expression of FAT/CD36, mCPT-I, and MCAD was completely abolished by addition of anti-TNF-α antibodies to the culture medium during the entire culture period (shown is protein expression on day 14). (B) Reduction of palmitate oxidation was abolished by anti TNF-α (values of day 14). *n*, at least four determinations for each culture interval and each condition. Values are means ± SEM. \**P* < 0.05 compared with control, †*P* < 0.05 compared with Ang II alone.

contrast to Ang II, TNF-α markedly reduced protein expression of the three PPAR isoforms during the entire culture period (Figures 5A-C).

In ARC exposed to Ang II, the addition of anti-TNF-α antibodies, or blocking of TNF-α synthesis by apocynin, NAC, or SN50, prevented the late decrease of PPARα, PPARβ/δ, and



**Figure 5** Effect of Ang II and TNF- $\alpha$  on protein expression of PPAR $\alpha$  (A), PPAR $\beta/\delta$  (B), and PPAR $\gamma$  (C). *n*, at least three determinations for each culture interval and each condition. (D) Inhibition of ROS accumulation by NAC or apocynin, blockade of nuclear translocation of NF- $\kappa$ B by SN50, or addition of anti-TNF- $\alpha$  antibodies prevented downregulation of all three PPAR isoforms (day 14 of culture). *n*, at least four determinations for each condition. Values are means  $\pm$  SEM. \**P* < 0.05 compared with control.

PPAR $\gamma$  proteins (Figure 5D). Collectively, the results suggest that protein expression of PPARs is reduced during prolonged exposure to Ang II by release of TNF- $\alpha$ , analogous to observations on FAT/CD36, mCPT-I, and MCAD. Ang II itself seems rather to enhance protein expression of PPARs and counteract the effect of TNF- $\alpha$ .

### 3.5 Activation of PPAR $\alpha$ and PPAR $\beta/\delta$ attenuates the effects of Ang II on the fatty acid oxidation pathway

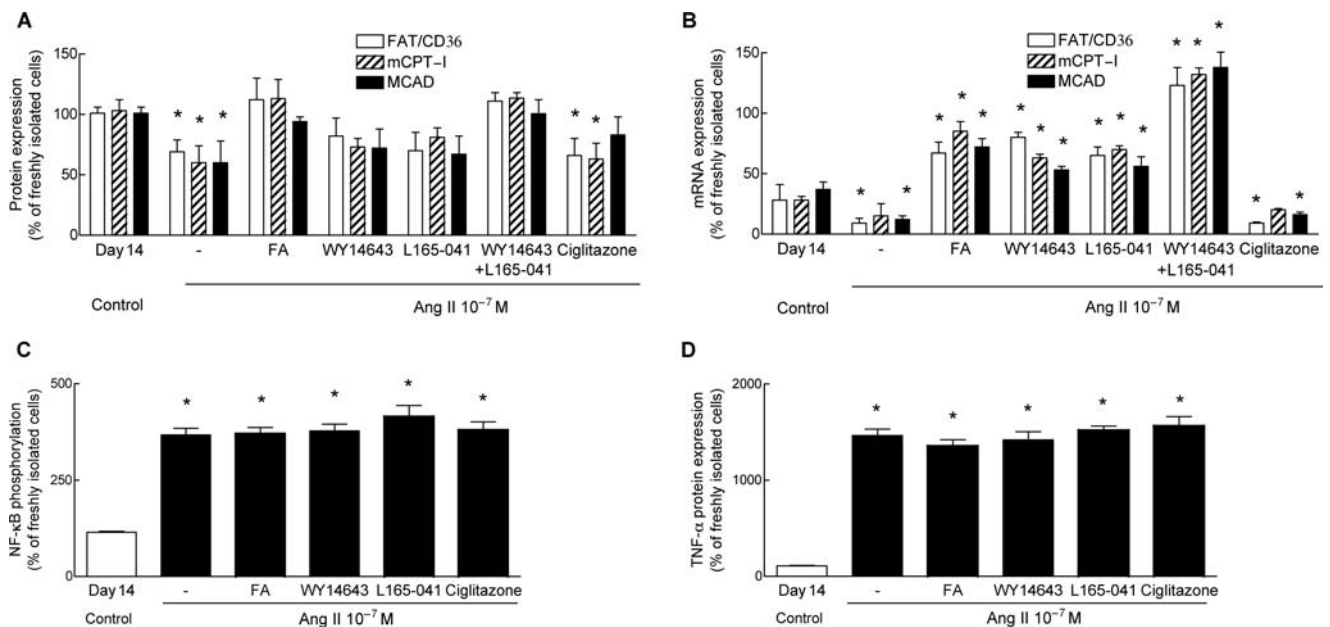
To determine whether ligand-mediated activation of PPARs influences Ang II-induced downregulation of the fatty acid oxidation pathway, we first examined the effect of supplementation of the culture medium with fatty acid.<sup>26</sup> In ARC exposed to Ang II (100 nM) supplementation of the medium with fatty acid, concentration-dependently attenuated the reduction of PPAR protein expression (Supplementary material online, Figure S4). The addition of 0.5 mM fatty acid (0.25 mM palmitate plus 0.25 mM oleate) completely prevented the Ang II-induced reduction of protein expression of all three PPAR isoforms (Supplementary material online, Figure S4). Fatty acid also prevented the reduction of protein expression of FAT/CD36, MCAD, and mCPT-I concentration-dependently (Figure 6A; Supplementary material online, Figure S4), while mRNA expression of regulatory proteins was increased four- to five-fold compared with ARC exposed to Ang II alone (Figure 6B).

To identify the role of individual PPAR isoforms, we supplemented the culture medium with specific agonists of either PPAR $\alpha$  (WY-14643), PPAR $\beta/\delta$  (L165-041) or PPAR $\gamma$  (ciglitazone) in fatty acid-free standard medium. Specificity of these ligands has been shown previously.<sup>26</sup>

Activation of either PPAR $\alpha$  by WY-14643 or of PPAR $\beta/\delta$  by L165-041 partially restored protein expression of FAT/CD36, mCPT-I, and MCAD, while combined stimulation of both isoforms completely restored protein expression (Figure 6A). mRNA expression was concomitantly increased 4- to 10-fold to values exceeding those measured in untreated control ARC (Figure 6B). In contrast, activation of PPAR $\gamma$  by ciglitazone had no effect on mRNA and protein expression of fatty acid oxidation genes. Neither fatty acid nor specific agonists prevented Ang II-mediated phosphorylation of NF- $\kappa$ B and enhancement of TNF- $\alpha$  protein expression (Figure 6C and D). The results indicate that combined activation of PPAR $\alpha$  and PPAR $\beta/\delta$  counteracts downregulation the fatty acid oxidation pathway by Ang II, independently of TNF- $\alpha$  synthesis, most likely by the stimulation of transcription of regulatory proteins of fatty acid oxidation.

### 3.6 Effect of inhibition Ang II-mediated generation of tumour necrosis factor- $\alpha$ and activation of PPARs on morphology and brain natriuretic peptide expression of adult rat cardiac

In Ang II-treated ARC, scavenging of ROS by NAC, inhibition of NF- $\kappa$ B translocation to the nucleus by SN50, or binding



**Figure 6** Activation of PPAR $\alpha$  and PPAR $\beta/\delta$ , but not of PPAR $\gamma$ , increased protein and mRNA expression of regulatory proteins of fatty acid oxidation without reduction of TNF- $\alpha$ . (A) Supplementation of the culture medium with fatty acid (FA; 50% palmitate/50% oleate) or with specific ligands of PPAR $\alpha$  (WY14643) or PPAR $\beta/\delta$  (L165-041) increased protein expression of FAT/CD36 (open bars), mCPT-I (hatched bars), and MCAD (filled bars). Activation of PPAR $\gamma$  by ciglitazone had little effect. (B) Fatty acid, WY14643, and L165-041 markedly increased mRNA expression of regulatory proteins to levels exceeding control. (C and D) Activation of PPARs did neither reduce Ang II-mediated phosphorylation of NF- $\kappa$ B (C), nor induction of TNF- $\alpha$  protein expression (D). Data of all panels were measured on day 14. *n*, at least four determinations for each condition. Values are means  $\pm$  SEM. \**P* < 0.05 compared with control.

of TNF- $\alpha$  by anti-TNF- $\alpha$  antibodies reduced cell surface and BNP mRNA to values comparable to untreated control ARC (Supplementary material online, *Figure S5*). Thus, interventions that prevent Ang II-mediated downregulation of the fatty acid oxidation pathway by inhibition of TNF- $\alpha$  expression also attenuate the hypertrophic response and BNP expression (Supplementary material online, *Figure S5*).

On the other hand, selective activation of PPAR $\alpha$  and PPAR $\beta/\delta$ , which prevent reduction of regulatory proteins of fatty acid oxidation, had no effect on cell size and BNP mRNA. However, selective activation of PPAR $\gamma$  and non-selective activation of all PPAR isoforms by fatty acids prevented the increase in cell surface area and upregulation of mRNA expression of BNP and ANF (Supplementary material online, *Figures S4* and *S5*) in ARC exposed to Ang II. Thus, in our study activity of PPAR $\gamma$ , but not activity of PPAR $\alpha$  and PPAR $\beta/\delta$  seem to modulate the hypertrophic phenotype induced by Ang II.

#### 4. Discussion

Ang II has been ascribed a central role in the phenotype modification of cardiac myocytes during pathological ventricular remodelling and heart failure. We have previously reported that failing hearts of mice with targeted overexpression of angiotensinogen in the myocardium exhibited marked downregulation of fatty acid oxidation enzymes.<sup>9</sup> Our present study indicates that prolonged exposure of ARC myocytes to Ang II is sufficient to reduce expression of key regulatory proteins of fatty acid oxidation including FAT/CD36, mCPT-I, and MCAD. This effect of Ang II on metabolic regulation is generated indirectly by stimulation of synthesis of TNF- $\alpha$  in the cardiac myocytes in response to ROS-induced activation of NF- $\kappa$ B.

The involvement of TNF- $\alpha$  in Ang II-mediated downregulation of the fatty acid oxidation pathway is supported by at least three observations of the present study: first, the onset of downregulation of regulatory proteins of fatty acid oxidation coincides with the onset of Ang II-induced generation of TNF- $\alpha$ . Second, the addition of TNF- $\alpha$  to the culture medium resulted in rapid reduction of protein expression of regulatory proteins of fatty acid oxidation. Third, addition of anti-TNF- $\alpha$  antibodies to Ang II-stimulated ARC completely blocked the effect of Ang II on the fatty acid oxidation pathway. To date, very little is known on the effects of TNF- $\alpha$  on metabolic regulation in the myocardium. Consistent with our results, Sekiguchi *et al.*<sup>31</sup> have observed in 8-week-old mice with cardiac-restricted overexpression of TNF- $\alpha$  that mRNA expression of FAT, mCPT-I, and very long-chain acyl-CoA dehydrogenase was reduced. Although protein expression has not been reported in their study, palmitate oxidation, measured in myocardial homogenate, was reduced, compatible with concomitant reduction of regulatory proteins.

Previous studies have demonstrated that TNF- $\alpha$  biosynthesis is stimulated in cardiac myocytes in response to Ang II.<sup>27</sup> In contrast to observations in adult feline cardiac myocytes cultured in serum-free medium,<sup>27</sup> we did not detect expression of TNF- $\alpha$  in ARC for 3 to 10 days after the onset of exposure to Ang II. The time-delay was dose-dependent and decreased with increasing concentration of Ang II. It needs to be emphasized that in the present study ARC were cultured with 20% FCS, which may alter the cellular response to Ang II. Using this culture condition, ARC undergo during the first 2 to 3 days a process of dedifferentiation with phenotypic changes, which include disassembly of the contractile elements,<sup>32</sup> followed by redifferentiation with resumption of contraction between day 4 and 7.<sup>33</sup> Ang II

signalling was not abrogated during the first week of culture, since Ang II-mediated phosphorylation of NF- $\kappa$ B and stimulation of protein synthesis were not abolished. Reduction of FCS to 1% shortened the time-delay of Ang II-induced reduction of regulatory proteins of fatty acid oxidation and palmitate oxidation rate, indicating that some component(s) of the serum may protect fatty acid oxidation from Ang II-induced downregulation. The mechanism remains unknown. Noteworthy, reduction of FCS to 1% did neither alter the ultimate extent of reduction of regulatory proteins of fatty acid oxidation and palmitate oxidation rate, nor the reversal by anti-TNF- $\alpha$  antibodies. Therefore, the concentration of FCS seems not critical for the ultimate effects of Ang II on the fatty acid oxidation pathway.

Consistent with observations in neonatal rat cardiac myocytes,<sup>19</sup> our results suggest a central role of activation of NF- $\kappa$ B in downregulation of the fatty acid oxidation pathway. Inhibition of Ang II-mediated activation or translocation of NF- $\kappa$ B by apocynin, NAC or SN50 completely prevented reduction of regulatory proteins of fatty acid oxidation. NF- $\kappa$ B has been proposed to lower fatty acid oxidation by direct interaction with PPARs which regulate transcription of target genes involved in fatty acid oxidation (transrepression).<sup>19</sup>

NF- $\kappa$ B is activated by receptor-initiated signalling cascades of both Ang II, via the AT-1 receptor,<sup>34</sup> and TNF- $\alpha$ , via TNF- $\alpha$  receptor 1.<sup>35</sup> Furthermore, Ang II-induced activation of NF- $\kappa$ B stimulates TNF- $\alpha$  synthesis in adult cardiac myocytes,<sup>27</sup> which may greatly amplify activation of NF- $\kappa$ B during exposure to Ang II. In fact, in our experiments, inactivation of TNF- $\alpha$  by anti-TNF- $\alpha$  antibodies did not diminish Ang II-induced phosphorylation of NF- $\kappa$ B on days 1 and 3, but markedly attenuated NF- $\kappa$ B phosphorylation on day 7 and later, indicating a predominant role of TNF- $\alpha$  in NF- $\kappa$ B activation during the second week of culture. The finding that inactivation of TNF- $\alpha$  by anti-TNF- $\alpha$  antibodies prevents downregulation of the fatty acid oxidation pathway suggests that activation of NF- $\kappa$ B by Ang II alone is sufficient to trigger synthesis of TNF- $\alpha$ , but insufficient to reduce levels of regulatory proteins of fatty acid oxidation. The cellular mechanism underlying the necessity of TNF- $\alpha$  for downregulation of the fatty acid oxidation pathway is open to speculation. On one hand, activation of NF- $\kappa$ B by stimulation of the AT-1 receptor alone may be quantitatively insufficient. In fact, TNF- $\alpha$ -independent activation of NF- $\kappa$ B by Ang II, estimated during inhibition of TNF- $\alpha$  by antibodies, was markedly lower compared with activation without TNF- $\alpha$  inhibition. On the other hand, downregulation of regulatory proteins of fatty acid metabolism may be mediated by NF- $\kappa$ B-independent signals triggered by TNF- $\alpha$ .

Considerable evidence implicates reduction of transcriptional activity of PPAR $\alpha$  and/or PPAR $\beta/\delta$  in the downregulation of the fatty acid oxidation pathway during progression of maladaptive myocardial remodelling.<sup>19</sup> Both PPAR $\alpha$  and PPAR $\beta/\delta$  are involved in the regulation of transcriptional activity of genes encoding regulatory proteins of fatty acid metabolism, including FAT/CD36, mCPT-I, and MCAD. Proposed mechanisms for inactivation of transcriptional activity of PPAR $\alpha$ , which is best characterized as yet, include reduced expression of PPARs,<sup>17</sup> posttranslational inactivation by Erk1/2-mediated phosphorylation,<sup>17</sup> reduced availability of cofactors including PGC-1,<sup>36</sup> and increased

expression of the repressor protein chicken ovalbumin upstream promoter transcription factor.<sup>18</sup> Finally, transcriptional activity may be reduced by transrepression by nuclear transcription factors including NF- $\kappa$ B and activator protein-1 (AP-1), as has been shown in phenylephrine-stimulated neonatal rat cardiac myocytes for PPAR $\beta/\delta$ .<sup>19</sup>

In our study, mRNA expression of FAT/CD36, mCPT-I, and MCAD was reduced by Ang II in ARC after 3 days of culture, indirectly suggesting that the transcription was decreased early during exposure to Ang II. Since protein expression of all three PPAR isoforms was decreased only during the second week of exposure to Ang II, the results are compatible with the interpretation that inactivation of PPARs preceded reduction of PPAR protein expression.<sup>17,18,36</sup> Despite early reduction of mRNA expression of FAT/CD36, mCPT-I, and MCAD, protein expression was maintained for several days and dropped only during the second week of culture, when TNF- $\alpha$  was generated. Therefore, it is likely that post-transcriptional mechanisms are critically involved in the regulation of protein expression of FAT/CD36, mCPT-I, and MCAD in ARC and the reduction in response to Ang II. The mechanism of presumed posttranscriptional reduction of regulatory proteins of fatty acid oxidation by TNF- $\alpha$  is not known. However, there is evidence suggesting that TNF- $\alpha$  may enhance degradation of selected proteins by the ubiquitin-proteasome pathway.<sup>37</sup>

We have previously observed a similar dissociation between myocardial mRNA and protein expression of regulatory proteins of fatty acid metabolism in mice with targeted overexpression of angiotensinogen in the myocardium.<sup>9</sup> mRNA expression of mCPT-I and MCAD progressively decreased during compensated hypertrophy, whereas protein expression did not change. However, protein expression and palmitate oxidation, measured during isolated heart perfusion *in vitro*, markedly dropped after the onset of heart failure. A similar observation has been reported by Sack *et al.*<sup>14</sup> in spontaneously hypertensive SHHR/Mcc-fa<sup>CP</sup> rats. Although myocardial TNF- $\alpha$  content has not been measured in these *in vivo* studies, our results raise the possibility that the reduction of protein expression of regulatory proteins of fatty acid oxidation and consecutive reduction of metabolic flux may be related to enhanced exposure of the myocardium to TNF- $\alpha$ .<sup>38</sup>

Although our data indirectly suggest the involvement of posttranslational mechanisms, the results clearly indicate that PPAR activity critically influences both the hypertrophic response and expression of regulatory proteins of fatty acid oxidation in ARC exposed to Ang II. Supplementation of the culture medium with 0.5 mM fatty acid (50% oleate/50% palmitate) and selective activation of PPAR $\alpha$  and PPAR $\beta/\delta$ , but not activation of PPAR $\gamma$ , restored mRNA and protein expression of regulatory proteins of fatty acid metabolism, and prevented reduction of palmitate oxidation rate.

Activation of PPAR $\alpha$  and PPAR $\beta/\delta$  may prevent reduction of regulatory proteins of fatty acid oxidation by at least two different mechanisms. The first mechanism is direct stimulation of transcription of target genes by binding to peroxisome proliferator responsive elements in the promoter region. Consistent with this possibility, mRNA expression of regulatory genes of fatty acid metabolism was increased 4- to 10-fold concomitantly with the restoration of protein expression. The second mechanism is direct interaction with transcription factors that are involved in the



hypertrophic response, including NF- $\kappa$ B and AP-1, which potentially may, directly or indirectly, contribute to reduction of proteins of fatty acid oxidation.<sup>39</sup> Consistent with this concept, some studies have provided evidence that PPAR $\alpha$  reduces TNF- $\alpha$  in the myocardium exposed to hypertrophic<sup>40</sup> or inflammatory stimuli,<sup>41</sup> apparently by inhibition of NF- $\kappa$ B.<sup>41</sup> However, in our study, we did not observe a reduction of TNF- $\alpha$  during specific or non-specific stimulation of either PPAR isoform in ARC exposed to Ang II. Therefore, our observations are more consistent with the interpretation that stimulation of transcription during activation of PPAR $\alpha$  and PPAR $\beta/\delta$  compensated for both transcriptional and posttranscriptional reduction of regulatory proteins of fatty acid oxidation. This mechanism of restoration of regulatory proteins of fatty acid metabolism differs from that effective during restoration of the fatty acid oxidation pathway by NAC, apocynin, or SN50, which all reduced synthesis of TNF- $\alpha$  by prevention of activation and nuclear translocation of NF- $\kappa$ B.

In summary, the findings of this study demonstrate for the first time that prolonged exposure of cardiac myocytes to Ang II may alter substrate metabolism in cardiac myocytes, characterized by reduction of the fatty acid oxidation capacity. The effect is largely mediated by the inflammatory cytokine TNF- $\alpha$ . The observation may be relevant for the understanding of the cellular mechanisms underlying the shift from fatty acid to glucose metabolism observed in the myocardium under conditions of ventricular remodeling and heart failure.<sup>7,9,10,14</sup> A critical question remains whether limitation of fatty acid oxidation is an adaptive response or may contribute to further damage of the myocardium.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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