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Angiotensin II and tumour necrosis factor α as mediators of ATP-dependent potassium channel remodelling in post-infarction heart failure

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Aims Angiotensin II (Ang II) and tumour necrosis factor α (TNFα) are involved in the progression from compensated hypertrophy to heart failure. Here, we test their role in the remodelling of ATP-dependent potassium channel (K_{ATP}) in heart failure, conferring increased metabolic and diazoxide sensitivity.
Methods and results We observed increased expression of both angiotensinogen and TNFα in the failing rat myocardium, with a regional gradient matching that of the K_{ATP} subunit Kir6.1 expression. Both angiotensinogen and TNFα expression correlated positively with Kir6.1 and negatively with Kir6.2 expression across the post-infarction myocardium. To further identify a causal relationship, cardiomyocytes isolated from normal rat hearts were exposed *in vitro* to Ang II or TNFα. We observed increased Kir6.1 and SUR subunit and reduced Kir6.2 subunit mRNA expression in cardiomyocytes cultured with Ang II or TNFα, similar to what was observed in failing hearts. In patch-clamp experiments, cardiomyocytes cultured with Ang II or TNFα exhibited responsiveness to diazoxide, in terms of both K_{ATP} current and action potential shortening. This was not observed in untreated cardiomyocytes and resembles the diazoxide sensitivity of failing cardiomyocytes that also overexpress Kir6.1. Ang II exerted its effect through induction of TNFα expression, because TNFα-neutralizing antibody abolished the effect of Ang II, and in failing hearts, regional expression of angiotensinogen matched TNFα expression. Finally, Ang II and TNFα regulated K_{ATP} subunit expression, possibly through differential expression of Forkhead box transcription factors.
Conclusion This study identifies Ang II and TNFα as mediators of the remodelling of K_{ATP} channels in heart failure.

1. Introduction

ATP-dependent potassium (K_{ATP}) channels are expressed at a high level in cardiomyocytes and play the role of metabolic sensor.[1–3] The opening of a potassium conductance in situations of low-energy supply reduces excitability of cardiomyocytes and shortens the action potential.[4] Activation of K_{ATP} channels in the heart is central to the cardioprotection against ischaemia afforded by preconditioning.[5,6] In addition, K_{ATP} channels were recently shown to play a key role in physiological stress situations such as intense exercise, as demonstrated by the observation that mice with genetically reduced cardiac K_{ATP} expression only tolerate half the level of exercise tolerated by wild-type mice.[7,8] Furthermore, these mice with genetically reduced cardiac K_{ATP} expression develop fulminant heart failure with impaired intracellular calcium control in response to haemodynamic overload.[9,10] Thus, K_{ATP} channels are also required to efficiently cope with stress in the context of an otherwise normal energy supply.

K_{ATP} channels are heteromultimeric assemblages of inwardly rectifying pore-forming subunits and regulatory subunits termed sulfonyl urea receptor. There are two isoforms of the conductance subunit, Kir6.1 or Kir6.2, and four isoforms of the regulatory subunits, SUR1A, SUR1B, SUR2A or SUR2B. Importantly, the physiological as well as the pharmacological properties of K_{ATP} channels depend on the constitutive subunits.[11,12] Kir6.1 channels have a considerably lower maximal unitary conductance when compared with Kir6.2 channels, but exhibit significant residual activity at rest.[13,14] Various SUR subunits confer distinct metabolic sensitivities to the complexes. Although

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electrophysiological evidences indicate that sarcolemmal K_{ATP} channels in ventricular cardiomyocytes are mostly Kir6.2/SUR2A,[15–17] immunological detection methods unambiguously showed Kir6.1 to be expressed as well in ventricular cardiomyocytes.[18,19]

We previously observed in post-infarction heart failure marked alterations of K_{ATP} subunits expression and electrophysiology. In short, the Kir6.1 conductance subunit and SUR regulatory subunits were overexpressed in the infarct border zone, conferring to failing cardiomyocytes responsiveness to the K_{ATP} opening drug diazoxide, which has little effect on normal ventricular cardiomyocytes.[20] An increased Kir6.1/Kir6.2 ratio may also render sarcolemmal K_{ATP} channels more sensitive to metabolic inhibition.[21] The molecular and cellular mechanisms responsible for these changes remain unknown. From the literature, overexpression of Kir6.1 has also been observed in ischaemic rat heart,[22] in which situation activation of the local renin–angiotensin system has been implicated.[23] On the other hand, we recently showed that expression of Kir6.1 and SUR subunits in post-infarction heart failure correlated positively with expression of the Forkhead box transcription factors FoxO3 and FoxF2 and negatively with that of FoxC2.[24]

We undertook the present study to determine whether exposure of cardiomyocytes to angiotensin II (Ang II) could reproduce the pattern of K_{ATP} subunits and Forkhead box transcription factors expression observed in post-infarction heart failure. Because the Ang II system is known to induce expression of tumour necrosis factor α (TNFα) in cardiomyocytes,[25] we also investigated the possible involvement of TNFα in the expression of K_{ATP} subunits and Forkhead box transcription factors.

2. Methods

An expanded Methods section is provided in the Supplementary material online.

2.1 Animal model of post-infarction heart failure

The Ethics Committee of the University of Geneva School of Medicine and the Geneva State Veterinary Office approved the study protocol, which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We have previously described induction of post-infarction heart failure in rats.[20] *In vivo* gene expression data presented in this study were obtained in samples collected in the previous study.

2.2 *In vitro* cultured cardiomyocytes

Adult rat cardiomyocytes were isolated and cultured as described previously.[26] The culture medium was M199 with Earle's salts (Life Technologies) supplemented with 20 mM creatine, 100 μM cytosine-β-D-arabinofuranoside, and 1% FCS. Ang II (100 nM) or recombinant rat TNFα (10 ng/mL ≈ 6×10^{-10} M; Life Sciences) was added at the time of plating (day 0). In some experiments, a neutralizing anti-rat TNFα antibody (150 ng/mL; Leinco Technologies) was also included at the time of plating. Cardiomyocytes were cultured in gelatin-coated 6 cm Petri dishes for gene expression determinations or on laminin-coated 24 mm round glass coverslips for patch-clamp experiments.

2.3 Gene expression analysis

mRNA expression was analysed by real-time RT–PCR according to standard protocols as described previously.[20,24] The appearance of PCR products was monitored either by the TaqMan probe method or by the SYBR Green method. Primers and probes for the real-time RT–PCR are given in Supplementary material online, *Table S1*.

2.4 Patch-clamp analysis

Whole-cell patch-clamp recording of K_{ATP} currents and action potentials were as described previously.[20]

2.5 Statistics

Data are presented as mean ± SEM and were compared by ANOVA (Prism 4, GraphPad Software) followed by Bonferroni's *post hoc* test. Differences were considered significant when P was <0.05.

3. Results

3.1 Regional expression of angiotensinogen and TNFα in post-infarction failing hearts correlates with Kir6 expression

Because Ang II and TNFα are two factors involved in the progression from compensated hypertrophy to heart failure, we examined whether regional expression of these factors would correlate with the previously described regional overexpression of Kir6.1 and SUR2. Both angiotensinogen and TNFα were overexpressed in post-infarction failing hearts (*Figure 1A* and *B*), with a regional heterogeneity matching that of Kir6.1 overexpression (*Figure 1C* and *D*). Angiotensinogen and TNFα expression correlated positively with Kir6.1 expression, and TNFα expression correlated negatively with Kir6.2 expression, across the remodelled post-infarction myocardium (*Table 1*). No significant correlation was observed between the expression of angiotensinogen or TNFα and that of any of the SUR subunits.

3.2 Ang II and TNFα induce Kir6.1 and SUR overexpression in cardiomyocytes

The above results prompted us to investigate whether Ang II or TNFα could induce expression of Kir6.1 in normal cardiomyocytes. Both Ang II and TNFα increased ANF mRNA expression and cell size in cultured cardiomyocytes (see Supplementary material online, *Figure S1*). In control cardiomyocytes, expression of all K_{ATP} subunits but SUR2A remained relatively constant over the 4 days of treatment and did not appreciably diverge from *ex vivo* values. Kir6.1 mRNA expression steadily increased and Kir6.2 mRNA rapidly dropped in cardiomyocytes exposed to either Ang II or TNFα (*Figure 2*). In response to Ang II, however, Kir6.2 mRNA recovered within 3 days. Similar to Kir6.1, SUR1A or SUR1B mRNA steadily increased along the course of exposure to Ang II or TNFα. Following an initial drop, SUR2B mRNA increased massively by day 4. Finally, SUR2A mRNA displayed a less consistent behaviour, with a tendency to decrease with time that was only modestly exacerbated in response to TNFα. Overexpression of Kir6.1 and SUR subunits in response to TNFα were confirmed at the protein levels (*Figure 3*). There was in addition a trend towards decreased Kir6.2 expression only in response to TNFα. The protein changes in response to Ang II were less pronounced.

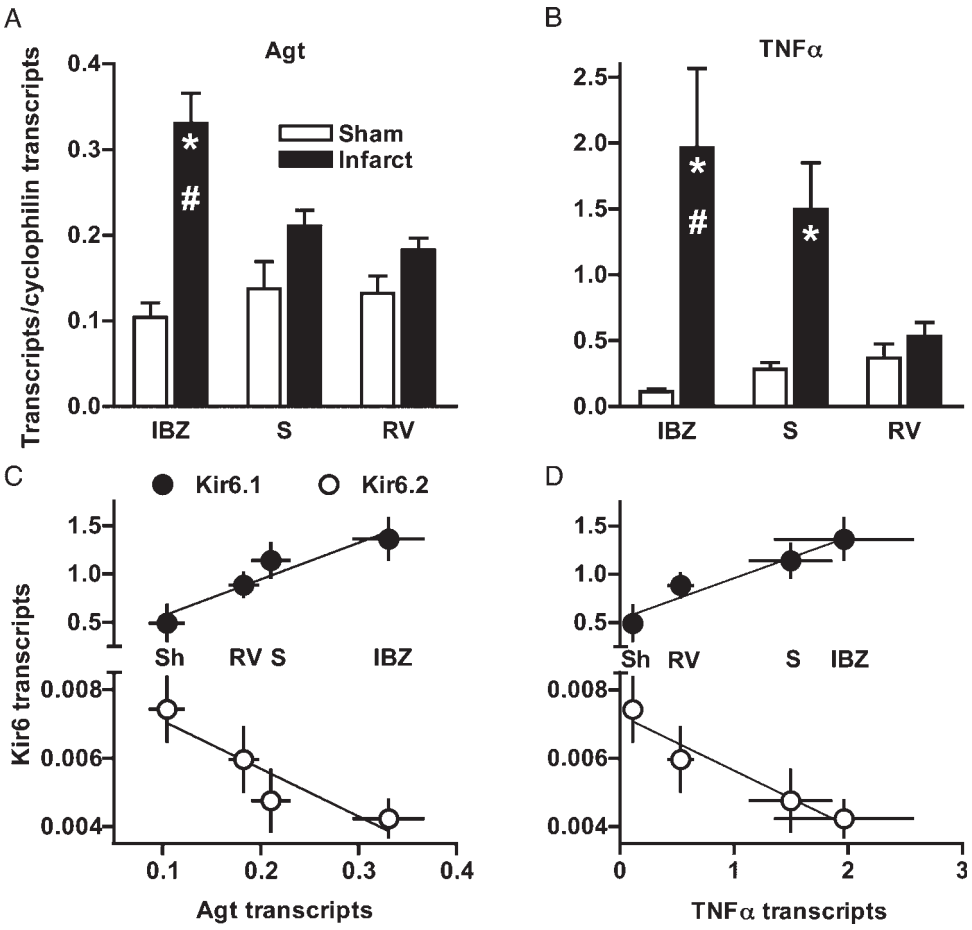

Figure 1 Regional expression of angiotensinogen and TNFα in post-infarction failing hearts correlates with Kir6 expression. (*A* and *B*) Total RNA was extracted from cardiomyocytes obtained from the infarct border zone (IBZ), the septum (S), or the right ventricle (RV). mRNA transcript number of angiotensinogen (Agt; *A*) and TNFα (*B*) was determined by quantitative RT–PCR in cardiomyocytes from sham (white bars, $n = 8$) or 20 weeks post-infarction failing hearts (black bars, $n = 11$) and normalized by the number of cyclophilin transcripts. *Significantly different from sham; #significantly different from the RV in failing hearts. (*C* and *D*) Expression of Kir6.1 and Kir6.2 (data from Isidoro Tavares *et al.*[20]) was correlated with that of Agt (*C*) or TNFα (*D*) in the various regions of 20 weeks post-infarction failing hearts. See *Table 1* for correlation parameters. Sh, pseudo-infarct border zone in sham hearts.

Table 1 Regional correlation between Kir6 and angiotensinogen and TNFα expression in post-infarction failing hearts

		Kir6.1	Kir6.2
Angiotensinogen	r^2	0.909	0.849
	P	0.047	0.079
TNFα	r^2	0.941	0.943
	P	0.003	0.029

Data from *Figure 1* and previous study.[20]

3.3 Changes in K_{ATP} currents reflect differential K_{ATP} subunits expression

Since pharmacological properties of K_{ATP} channels depend on the molecular channel composition,[11,12] we checked whether the changes in K_{ATP} subunit expression were matched by changes in K_{ATP} currents ($I_{K_{ATP}}$). Diazoxide opens a subset of K_{ATP} channels normally not observed in the sarcolemma of ventricular cardiomyocytes (Kir6.1/SUR2B, Kir6.1/SUR1, and Kir6.2/SUR1). P1075 opens all sarcolemmal K_{ATP} channels but the Kir6.1/SUR1 combination, whereas glibenclamide blocks all K_{ATP} channels regardless of the channel composition.

The basal current was larger in cardiomyocytes treated with TNFα when compared with control or Ang II-treated cardiomyocytes (*Figure 4*). P1075 increased $I_{K_{ATP}}$ in all the cardiomyocytes recorded and glibenclamide completely reversed the effect of P1075. The $I_{K_{ATP}}$ observed under P1075 stimulation was slightly larger in cardiomyocytes treated with Ang II or TNFα than in control cardiomyocytes. Most importantly, diazoxide stimulated $I_{K_{ATP}}$ only in cardiomyocytes exposed to either Ang II or TNFα. This effect appeared more pronounced with Ang II. However, further analysis of the TNFα-treated cardiomyocytes revealed that diazoxide could not evoke $I_{K_{ATP}}$ in all cells exposed to TNFα. Arbitrarily splitting the TNFα-treated cardiomyocytes into two groups according to cell size, with the cut-off set at 140 pF (2SD above the mean of control cardiomyocytes), showed that small cardiomyocytes (six of 15 analysed cells) were undistinguishable from control cardiomyocytes, whereas larger cardiomyocytes (nine of 15) had larger basal currents and their response to diazoxide was as great as the response of cardiomyocytes treated with Ang II (see Supplementary material online, *Figure S2*).

The primary physiological consequence of K_{ATP} opening in cardiomyocytes is a shortening of the action potential. Accordingly, we recorded action potentials in cardiomyocytes cultured in the presence of TNFα and acutely exposed to K_{ATP} openers (see Supplementary material online, *Figure S3*).

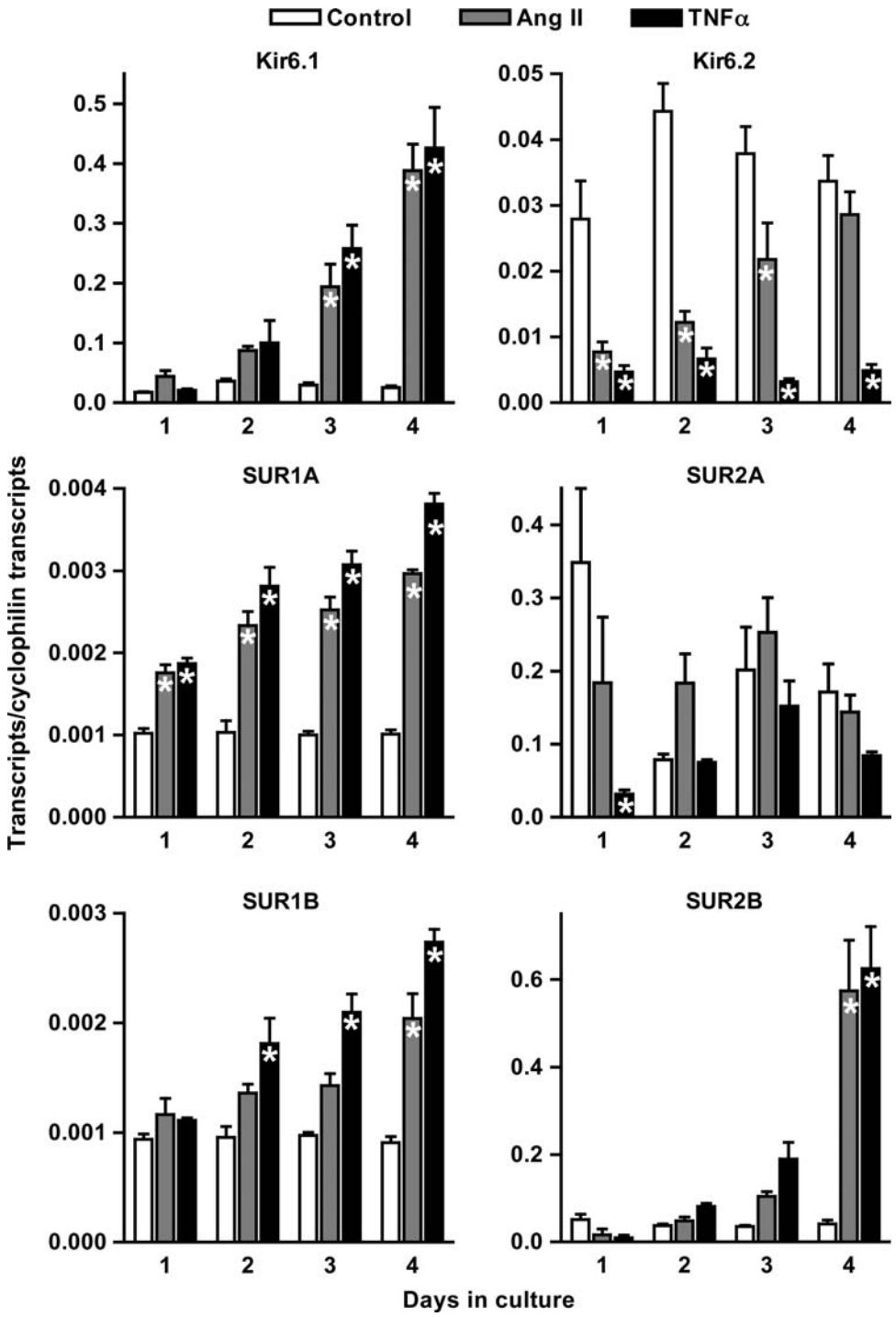

Figure 2 mRNA expression of K_{ATP} subunits in cardiomyocytes exposed to Ang II or TNFα. RT–PCR analysis of K_{ATP} subunits expression. Normal adult rat cardiomyocytes were exposed *in vitro* to either Ang II (100 nM; grey bars), TNFα (0.6 nM, black bars), or vehicle (white bars) for up to 4 days. Following total RNA extraction, the mRNA transcript numbers of Kir6.1, Kir6.2, SUR1A, SUR1B, SUR2A, and SUR2B were determined by quantitative RT–PCR and normalized by the number of cyclophilin transcripts. *Significantly different from control cardiomyocytes; $n = 4$–8.

Action potential shapes and durations were similar in cultured and *ex vivo*[20] control cardiomyocytes, suggesting that no major electrical remodelling took place during the culture period in control conditions. In the absence of K_{ATP} openers, triggered action potentials were longer in TNFα-treated cardiomyocytes, possibly because of reduced I_{to} and I_{Ks} expression.[27–29] Diazoxide shortened action potentials in TNFα-treated cardiomyocytes but had no effect on control cardiomyocytes; P1075 shortened action potentials in all cardiomyocytes. Similar to the *in vivo* situation, TNFα-treated cardiomyocytes with longer action potentials exhibited more pronounced effects of diazoxide.

3.4 TNFα expression mediates the effects of Ang II on expression of K_{ATP} subunits

Further analysis of the angiotensinogen and TNFα expression patterns in post-infarction failing myocardium revealed

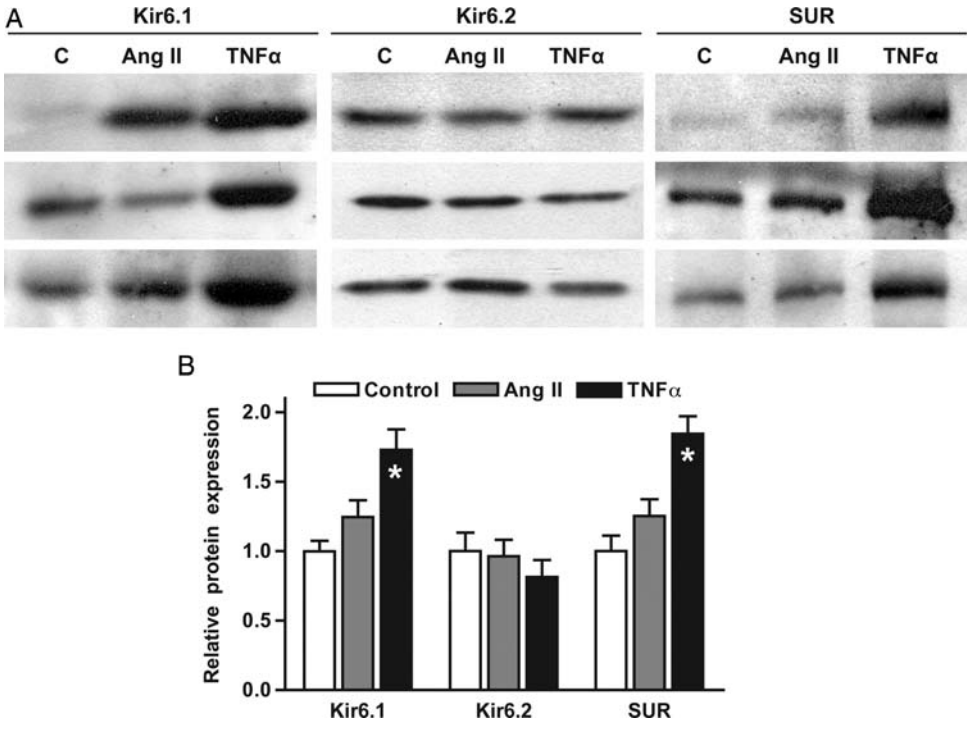

Figure 3 Protein expression of K_{ATP} subunits in cardiomyocytes exposed to Ang II or TNFα. Normal adult rat cardiomyocytes were exposed *in vitro* to Ang II (100 nM; grey bars), TNFα (0.6 nM, black bars), or vehicle (C; white bars) for 4 days. (*A*) Representative western blots for Kir6.1, Kir6.2, and SUR proteins. The antibody used for SUR western blot analysis does not discriminate between the various SUR isoforms. (*B*) Semi-quantitative analysis of Kir6.1, Kir6.2, and SUR proteins expression. *Significantly different from control cardiomyocytes; $n = 13–19$.

a correlation between mRNA expression of these two factors (*Figure 5A*). Furthermore, we confirmed the observation[25] that normal cardiomyocytes exposed *in vitro* to Ang II expressed more TNFα mRNA than control cardiomyocytes, although TNFα mRNA expression tended to rise spontaneously in cultured cardiomyocytes (*Figure 5B*).

To find out whether TNFα expression was important for the effects of Ang II, we co-incubated cardiomyocytes with Ang II and a TNFα-neutralizing antibody. As a positive control for the effect of the neutralizing antibody, it was also added in combination with TNFα. Addition of the TNFα-neutralizing antibody abolished all the effects of Ang II on expression of K_{ATP} subunits by day 4, and blunted the effects of TNFα (*Figure 6*). The TNFα-neutralizing antibody did not change the expression of K_{ATP} subunits in control cardiomyocytes.

3.5 Ang II and TNFα modify expression of Forkhead transcription factors

We recently described the overexpression in the post-infarction failing myocardium of the Forkhead transcription factors FoxO3 and FoxF2,[24] which regulate expression of K_{ATP} subunits. Similarly, the mRNA expression of FoxO3 and FoxF2 was markedly increased in cardiomyocytes exposed *in vitro* to either Ang II or TNFα (*Figure 7*); again, these overexpressions were blunted in the presence of the TNFα-neutralizing antibody. In contrast to FoxO3 and FoxF2, two other Forkhead transcription factors, FoxO1 and FoxC2, were down-regulated in response to Ang II or TNFα. This down-regulation was also dependent on TNFα, as indicated by its relief by the TNFα-neutralizing antibody. Finally, FoxJ2 showed an inconsistent but TNFα-dependent regulation over the 4 days of culture (see Supplementary material online, *Figure S5*; *Figure 7*).

4. Discussion

We had previously observed in post-infarction heart failure a remodelling of K_{ATP} channels expression in the left ventricle infarct border zone.[20] In the present study, we demonstrate for the first time the important role of TNFα in the regulation of K_{ATP} channels expression, mediating the activation of the local renin–angiotensin system. *In vivo* and *in vitro* observations indicate that the Ang II–TNFα relay can explain the remodelling of K_{ATP} channels in heart failure. Further, we provide indirect evidence that Ang II and TNFα regulate K_{ATP} subunits expression through alterations of the expression of Forkhead box transcription factors. Finally, the *in vitro* model used, replicating the *in vivo* K_{ATP} phenotype in heart failure, is valid to study the molecular mechanisms governing K_{ATP} channels expression in heart failure.

4.1 Ang II and TNFα as factors responsible for K_{ATP} expression alterations *in vivo*

The regional pattern of K_{ATP} expression remodelling in the post-infarction failing myocardium suggests that the factor(s) responsible for these changes had to be either locally present or only locally active. Both the renin–angiotensin system[30] and TNFα[31,32] match the first criterion. In addition, the involvement of the renin–angiotensin system in the overexpression of Kir6.1 in the ischaemic myocardium had already been reported.[23] Analysis of the regional expression of angiotensinogen and TNFα in post-infarction failing hearts revealed positive correlations with Kir6.1 expression and

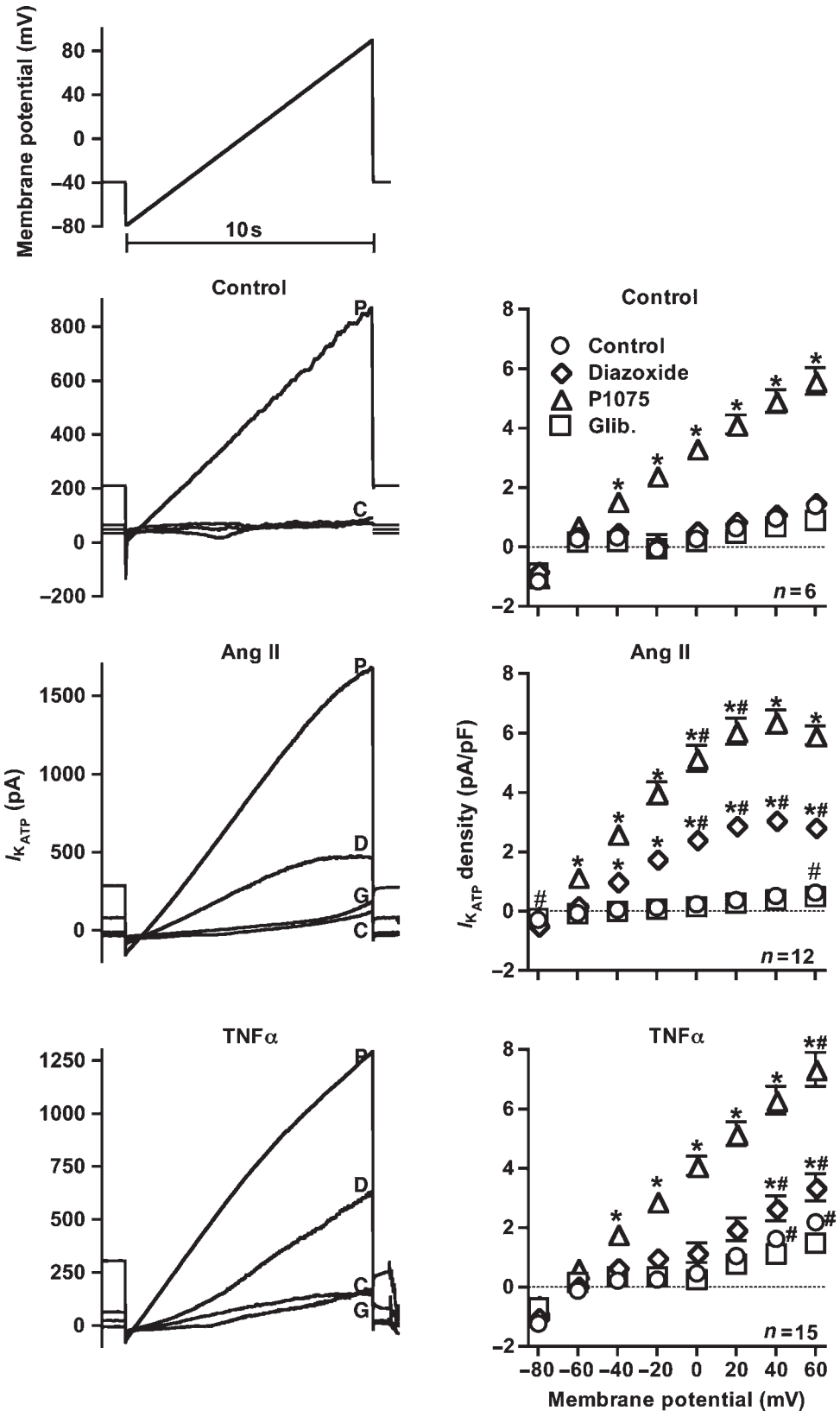

Figure 4 K_{ATP} currents ($I_{K_{ATP}}$) in cardiomyocytes exposed to Ang II or TNFα. Top left panel shows the voltage ramp protocol used for recording of K_{ATP} currents. Left: typical $I_{K_{ATP}}$ traces and right: mean $I_{K_{ATP}}$ density (current divided by cell capacitance) in cardiomyocytes exposed *in vitro* to Ang II, TNFα, or vehicle for 4 days. $I_{K_{ATP}}$ was recorded in the absence of drug (C; circles) or in the presence of diazoxide (D; 30 μM, diamonds), P1075 (P; 100 μM, triangles), or glibenclamide (G; 1 μM, squares). *Significantly different from $I_{K_{ATP}}$ density measured in the absence of drug; #significantly different from $I_{K_{ATP}}$ density measured in control cardiomyocytes.

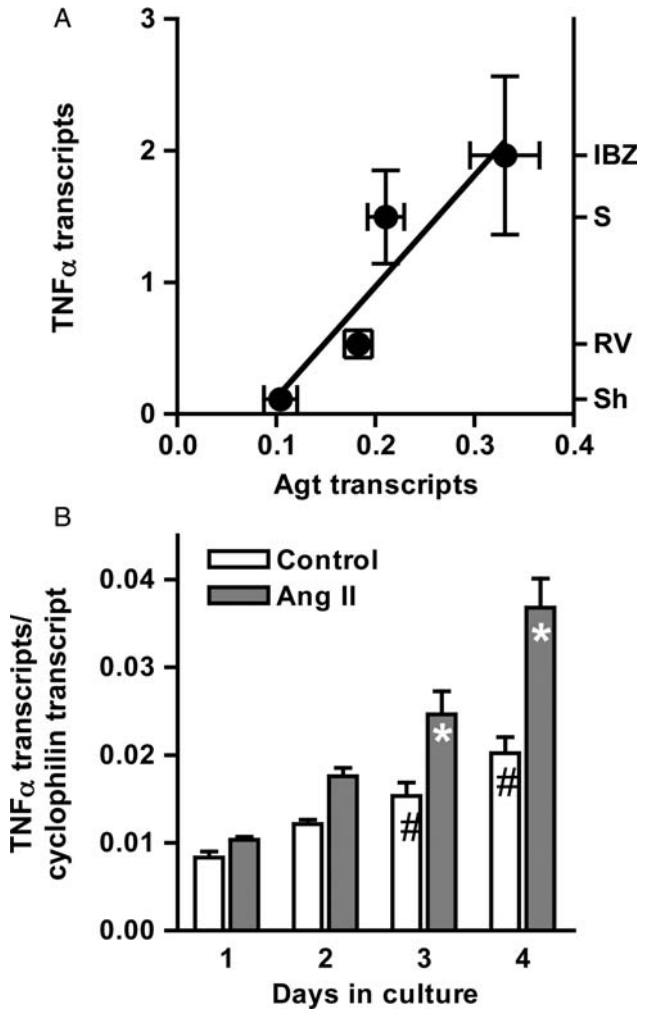

Figure 5 Ang II induces expression of TNFα in post-infarction failing hearts and *in vitro*. (*A*) Coexpression of TNFα and Agt in the various regions of 20 weeks post-infarction failing hearts (data from *Figure 1*). Correlation parameters: $r^2 = 0.87$, $P = 0.069$. IBZ, infarct border zone; S, septum; RV, right ventricle; Sh, pseudo-infarct border zone in sham hearts. (*B*) Normal adult rat cardiomyocytes were exposed *in vitro* to either Ang II (100 nM; grey bars) or vehicle (white bars) for 4 days. Following total RNA extraction, the mRNA transcript number of TNFα was determined by quantitative RT–PCR and normalized by the number of cyclophilin transcripts. *Significantly different from control cardiomyocytes; #significantly different from control at day 1; $n = 4$.

negative correlations with Kir6.2 expression. Although correlation is not a causal relationship and may arise from a common cause, further *in vitro* experiments in which exposure of normal cardiomyocytes to Ang II or TNFα resulted in identical changes in Kir6 expression support the conclusion that Ang II and TNFα are factors responsible for the altered expression of Kir6 subunits in heart failure *in vivo*. The participation of other locally produced or present factors, such as tissue hypoxia[33] or endothelin-1,[34] cannot be excluded. Indeed, exposure of normal cardiomyocytes to endothelin-1 *in vitro* produced similar, but less pronounced, changes in Kir6 expression as Ang II or TNFα (data not shown).

The absence of correlation between the *in vivo* regional expression of angiotensinogen or TNFα and that of SUR subunits should not lead to the conclusion that Ang II or TNFα do not impact on SUR expression in cardiomyocytes. In fact, *in vitro* experiments show marked effects of Ang II or TNFα on SUR mRNA and protein expression. Therefore, it is more likely that additional, as yet unidentified, factors also contribute to the regulation of SUR expression *in vivo*.

4.2 TNFα expression mediates the effects of Ang II

Observations of the present study suggest that most effects of Ang II on expression of K_{ATP} subunits are mediated by the paracrine or autocrine expression of TNFα. This conclusion is indirectly supported by data showing good correlation between the *in vivo* regional expression of angiotensinogen and that of TNFα. Most conclusively, the effects of Ang II *in vitro* on K_{ATP} subunits expression are essentially abrogated in the presence of a TNFα-neutralizing antibody. That the TNFα-neutralizing antibody less efficiently curtailed the effects of TNFα itself is readily explained by an insufficient titre of the antibody (150 ng/mL, i.e. ∼1 nM) to quell the high concentration of TNFα used (10 ng/mL, i.e. ∼0.6 nM), as the manufacturer recommends a 6:1 antibody:TNFα stoechiometry.

However, one effect of Ang II—the reduction of Kir6.2 expression—cannot be explained by TNFα expression. Although mostly extinct by day 4, this effect is very prominent on days 1–2 (*Figure 2*) and is then not diminished by the TNFα-neutralizing antibody (see Supplementary material online, *Figure S5*). Therefore, Ang II directly impacts on Kir6.2 expression independently of TNFα expression. That this effect disappears after 3–4 days might be due to tachyphylaxis,[35] whereas TNFα production is insufficient to mimic the effects of 10 ng/mL exogenous TNFα.

Indirect evidence suggesting that Ang II may have TNFα-independent effects on K_{ATP} expression and function arises from the minor discrepancy between Kir6.1 and SUR expression, which is highest under TNFα, and diazoxide responsiveness, which on average is highest under Ang II. Furthermore, a subset of cardiomyocytes exposed to TNFα did not modify their K_{ATP} electrophysiological profile (see Supplementary material online, *Figure S3*), whereas all cardiomyocytes exposed to Ang II did. The reasons for these differences remain unknown.

4.3 Role of Forkhead box transcription factors

A recent study from our laboratories has identified Forkhead box transcription factors (Fox) as important regulators of K_{ATP} subunits expression in cardiomyocytes.[24] In the setting of post-infarction heart failure, Kir6.1 expression was positively correlated with FoxF2 and FoxO3 expression, and negatively with FoxC2 expression, whereas Kir6.2 expression correlated positively with FoxO1 expression. FoxF2 expression appeared to positively influence expression of SUR subunits. Strikingly, in cardiomyocytes exposed to Ang II or TNFα, we observed a pattern of Fox expression that almost exactly matches the correlations from our previous study, with the exception of FoxJ2. Therefore, it is tempting to ascribe Kir6.1 up-regulation in the *in vitro* model to the combination of FoxF2 and FoxO3 overexpression and FoxC2 repression, whereas Kir6.2 down-regulation would result from FoxO1 repression. The causal relationship between Fox and K_{ATP} subunits expression has been demonstrated in neonatal cardiomyocytes and the HL-1 atrial cell line;[24] it remains to be worked out in adult ventricular myocytes. Intriguingly, whereas FoxF2 expression correlates positively with SUR2A and SUR2B expression in post-infarction heart failure, overexpression of FoxF2 in HL-1 cardiomyocytes

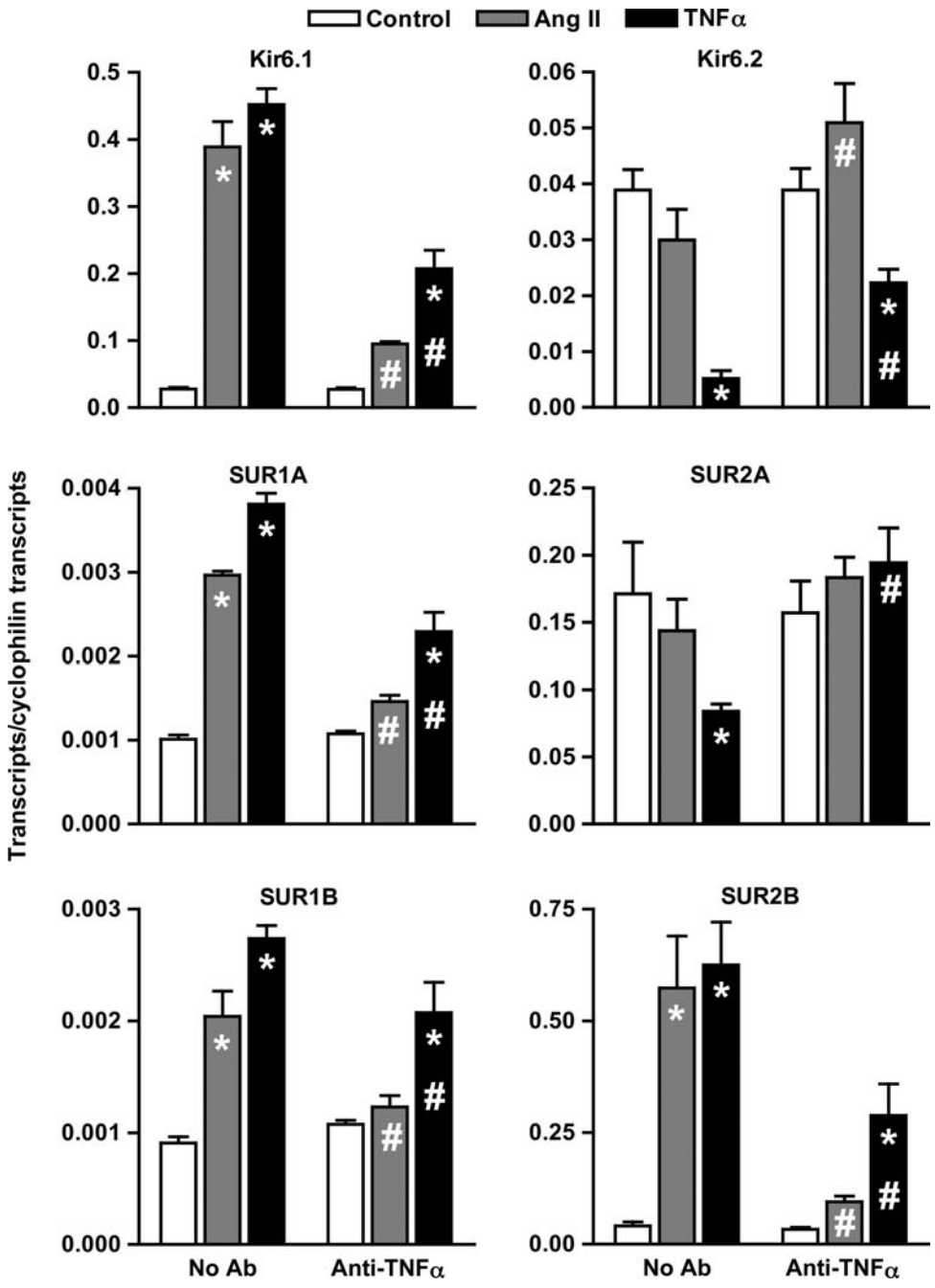

Figure 6 Incubation with a TNFα-neutralizing antibody abolishes the effects of Ang II on the expression of K_{ATP} subunits. Normal adult rat cardiomyocytes were exposed *in vitro* to Ang II (100 nM; grey bars), TNFα (10 ng/mL, black bars), or the vehicle (white bars) for 4 days in the presence or absence of a TNFα-neutralizing antibody. Following total RNA extraction, the mRNA transcript numbers of Kir6.1, Kir6.2, SUR1A, SUR1B, SUR2A, and SUR2B were determined by quantitative RT–PCR and normalized for the number of cyclophilin transcripts. *Significantly different from control cardiomyocytes; #significantly different from cardiomyocytes exposed to the same agonist in the absence of the TNFα-neutralizing antibody; $n = 4$.

results in stimulation of the expression of only SUR2A. In this study, only SUR2B expression is increased concomitantly with that of FoxF2. Thus clearly, the relationship between Fox transcription factors and K_{ATP} subunits expression is complex and not univocal.

Changes in Fox expression cannot explain all changes in K_{ATP} subunits expression. Indeed, in response to Ang II, SUR1A increases before FoxF2 and Kir6.2 drops before FoxO1 (*Figure 2* and see Supplementary material online, *Figure S6*), thus suggesting Fox-independent impacts of Ang II on expression of these subunits. Furthermore, despite statistical association, no FoxF2-binding site has been identified in the promoter region of the SUR1 gene.[24] The early SUR1A increase could, however, perhaps be ascribed to transient overexpression of FoxJ2 (see Supplementary material online, *Figure S5*), with which statistical association was observed *in vivo*.

4.4 *In vitro* replication of the K_{ATP} phenotype in failing hearts

A remarkable finding of this study is the almost exact replication of the K_{ATP} phenotype of failing cardiomyocytes from the infarct border zone through exposure *in vitro* to a single

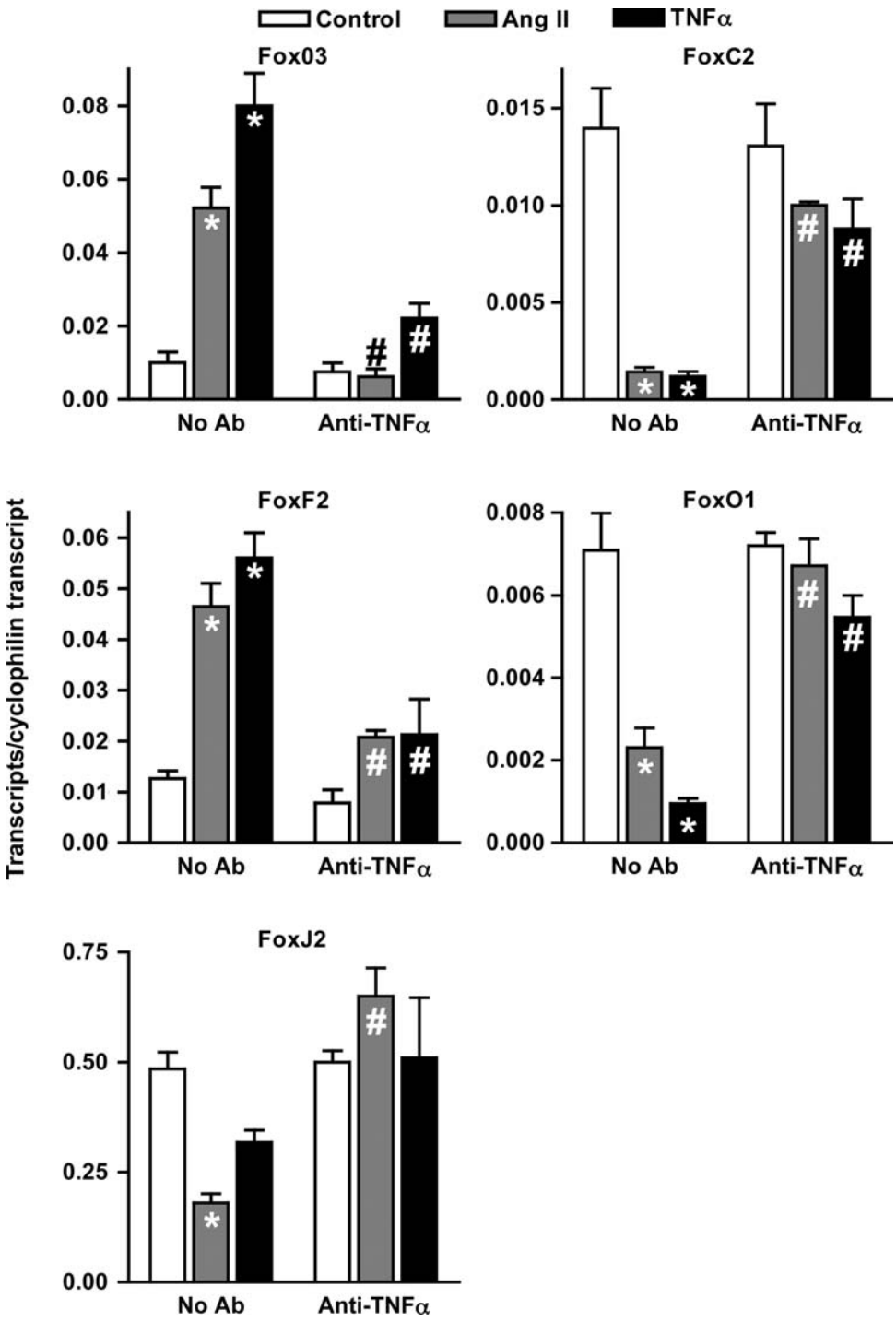

Figure 7 Expression of Forkhead transcription factors in cardiomyocytes exposed to Ang II or TNFα. Normal adult rat cardiomyocytes were exposed *in vitro* to Ang II (100 nM; grey bars), TNFα (10 ng/mL, black bars), or the vehicle (white bars) for 4 days in the presence or absence of a TNFα-neutralizing antibody. Following total RNA extraction, the mRNA transcript numbers of FoxO3, FoxF2, FoxC2, FoxO1, and FoxJ2 were determined by quantitative RT–PCR and normalized by the number of cyclophilin transcripts. *Significantly different from control cardiomyocytes; #significantly different from cardiomyocytes exposed to the same agonist in the absence of the TNFα-neutralizing antibody; $n = 4$.

factor, either Ang II or TNFα. The replication manifests itself through mRNA expression, protein expression, electrophysiology, and pharmacology. The less pronounced changes in protein expression in the Ang II-treated cardiomyocytes when compared with the TNFα-treated group could be related to a shorter exposure to a significant dose of TNFα, as this cytokine overexpression in response to Ang II is time-dependent (*Figure 5*). Interestingly, both *in vivo*[20] and *in vitro*, the reduction in Kir6.2 mRNA was not accompanied by a reduction of the Kir6.2 protein. A similar dissociation between reduced mRNA expression and sustained protein expression has been observed for proteins of the fatty acid oxidation pathway in *in vivo*[36,37] and *in vitro*[38,39] models of pathological myocardial hypertrophy. The mechanisms responsible for this dissociation remain unknown.

The notable deviations in the *in vitro* system from the *in vivo* model are in the expression of SUR2A and FoxJ2. SUR2A mRNA is unaffected by Ang II and tends to be reduced in response to TNFα in the present study, whereas it was uniformly increased in post-infarction myocardium *in vivo*.[20] This is all the more surprising as SUR2A is a splice variant of SUR2B, which is the most markedly increased mRNA in response to Ang II or TNFα. Thus, despite sharing most if not all SUR2 gene regulatory sequences, SUR2A and SUR2B splice variants exhibit inverse responsiveness to the same agonists, possibly through differential regulation of mRNA stability. FoxJ2 is reduced by Ang II and to a lesser extent by TNFα in the present study, whereas it was uniformly increased in post-infarction myocardium *in vivo*.[24] Intriguingly, it seems that both TNFα-dependent and -independent signalling are required for FoxJ2 down-regulation as it is most pronounced in response to Ang II but prevented by co-incubation with a TNFα-neutralizing antibody.

We could not verify whether the diverging mRNA expression of SUR2A and SUR2B translate into reduced SUR2A protein and increased SUR2B protein, because isoform-specific SUR antibodies were unsatisfactory. Western blot analysis with a pan-SUR antibody could only indicate a global increase in SUR subunits protein expression (*Figure 3*).

4.5 Molecular remodelling of sarcolemmal K_{ATP} channels in response to Ang II and TNFα

We observed a significant up-regulation of the conductance subunit Kir6.1 and all SUR but SUR2A subunits in cardiomyocytes exposed to either Ang II or TNFα. Together with the down-regulated Kir6.2 expression, these data suggest that the molecular composition of the sarcolemmal K_{ATP} channels changed to include more Kir6.1-containing complexes. Sarcolemmal K_{ATP} channels in normal cardiomyocytes are unresponsive to diazoxide,[20] and so is the Kir6.2/SUR2A assemblage when heterologously expressed.[11] $I_{K_{ATP}}$ in Ang II- or TNFα-treated cardiomyocytes can be stimulated by diazoxide and P1075. This pharmacological profile, together with gene expression data, is compatible with Kir6.1/SUR2B being an important component of sarcolemmal K_{ATP} channels in Ang II- or TNFα-treated cardiomyocytes, as Kir6.1/SUR1 K_{ATP} channels are unresponsive to P1075.[11] Nevertheless, we cannot rule out the existence of K_{ATP} channels with heteromeric Kir6.1/Kir6.2 pores, as such hybrid K_{ATP} channels have been shown to be responsive to diazoxide when co-assembled with SUR2B.[40]

4.6 Study limitations

Several limitations of the present study need to be discussed.

First, in the *in vivo* model of post-infarction, cardiomyocytes were isolated from ventricular regions according to the position of the infarct scar. In contrast, cardiomyocytes for *in vitro* culture in the presence of Ang II or TNFα were obtained indistinctly from all ventricular regions. Because reports have demonstrated a larger density of $I_{K_{ATP}}$ currents in the epicardium when compared with the endocardium,[41,42] single-cell data from patch-clamp experiments could potentially be biased. Such a bias would not exist in gene expression data, which results from the pooling of thousands of cardiomyocytes for each condition.

Secondly, numerous observations suggest that the expression of angiotensinogen and TNFα in the myocardium accompanies the transition from compensated hypertrophy to heart failure, the causal relationship remaining unclear.[43,44] This is a chronic, slowly evolving process. In contrast, the *in vitro* model described herein replicates, from a paracrine point of view, only the final stage by abruptly exposing cardiomyocytes to high concentrations of cytokines. To the best of our knowledge, none knows the actual concentrations of Ang II or TNFα in the interstitial fluid in failing myocardium. We therefore selected concentrations previously reported in the literature to be active on isolated cultured cardiomyocytes.

Thirdly, K_{ATP} currents were evoked in cultured cardiomyocytes by pharmacological openers, not metabolic stress, and in clamped ATP and ADP conditions (see Supplementary material online, Methods). Thus, potential alterations of ATP or ADP sensitivity of the K_{ATP} channels might have been overlooked. Indeed, reports in hypertrophied feline cardiomyocytes have indicated a reduced sensitivity of K_{ATP} to ATP.[45,46] In contrast, in cardiomyocytes from TNFα-induced failing mouse hearts, the intrinsic ATP sensitivity of the channels K_{ATP} was unaltered;[47] the cardiomyocytes showed reduced metabolic sensitivity because of impaired metabolic signal generation due to CK deficiency.

Finally, the evidence obtained in this study linking the regulation of Forkhead transcription factor expression with that of K_{ATP} subunits is only correlative. It would have been difficult to obtain more mechanistic data, as adult rat cardiomyocytes are not amenable to transfection, for example, with Fox expression vectors or Fox siRNAs. However, the data obtained concur quite substantially with information previously obtained in the HL-1 atrial cell line.[24]

4.7 Conclusions

In conclusion, this study identifies Ang II and TNFα as factors mediating the remodelling of K_{ATP} channels in the infarct border zone, most probably through alterations of Fox expression. The *in vitro* system described herein will be very valuable in the study of the molecular mechanisms governing K_{ATP} channels expression in heart failure.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Conflict of interest: none declared.

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