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Low Over-Expression of TNFα in the Mouse Heart Increases Contractile Performance Via TNFR1

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ABSTRACT

TNFα is a cytokine wit pleiotropic functions in many organs. In the heart increased TNFα levels are not only associated with heart failure, but also, paradoxically, with protection from ischemic damage. To test whether the protective role of TNFα in the heart is concentration-dependent, we studied two mouse heart models with low (two- to threefold) over-expression of endogenous TNFα: mice deficient in a translational repressor of TNFα mRNA, TIA-$1^{-/-}$, and mice over-expressing human TNFα. Hearts lacking TIA-1 were characterized for their endogenous TNFα over-expression during normal Langendorff perfusion. To define which TNFα receptor mediates cardiac protection, we also used mice lacking the TNFR1 receptor. Contractile function was assessed in isolated hearts perfused in the isovolumic Langendorff mode during and following global no-flow ischemic stress and in response to varying extracellular [Ca^{2+}] to determine their contractile response and Ca^{2+} sensitivity. All hearts with low over-expression of TNFα, independent of human or murine origin, have improved contractile performance and increased Ca^{2+} sensitivity (by 0.2–0.26 pCa). Hearts lacking TNFR1 have contractile performance equal to wild type hearts. Recovery from ischemia was greater in TIA-$1^{-/-}$ and was diminished in TNFR$1^{-/-}$. Better contractile function in TNFα over-expressing hearts is not due to improved cardiac energetics assessed as [ATP] and glucose uptake or to differences in expression of SERCA2a or calmodulin. We suggest that low levels of TNFα increase the Ca^{2+} sensitivity of the heart via a TNFR1-mediated mechanism. J. Cell. Biochem. 105: 99–107, 2008.

KEY WORDS: MYOCARDIAL CONTRACTION; CONTRACTILITY; TNFα; CA^{2+} SENSITIVITY

The cytokine tumor necrosis factor-alpha (TNFα) is produced by various cells including macrophages, adipocytes and cardiomyocytes [Kapadia et al., 1995a]. In the heart, TNFα mediates many effects, including negative inotropy, that is, the decrease in contractile strength [Yokoyama et al., 1993; Kapadia et al., 1995b], induction of apoptosis [Saraste et al., 1997; Haudek et al., 2007], and protection from ischemia-reperfusion injury [Kurrelmeyer et al., 2000; Lecour et al., 2002]. The mechanisms by which TNFα mediates these effects are not fully understood.

Elevated serum TNFα levels ($\sim 10^{-9}$ M) have been associated with the progression of heart failure in patients. This concentration of TNFα has negative inotropic effects on cardiomyocytes in vitro, and leads to heart failure in mice [Mann, 2003]. In contrast, a lower concentration ($\sim 10^{-11}$ M) of soluble TNFα has been found to protect mouse hearts from ischemia-reperfusion injury [Lecour et al., 2002]. These findings raise the possibility that TNFα can have either beneficial or detrimental effects on the heart that depend on its concentration. To test this hypothesis, we compared indices of cardiac contractile performance for wildtype (WT) mouse hearts and hearts isolated from two genetically modified mouse models that over-express low-levels of TNFα protein. The mouse models studied were chronic low over-expression of human TNFα protein in vivo (hTNF$\alpha^{+/+}$) and acute low over-expression of endogenous TNFα in mice deficient in a translational repressor of TNFα mRNA, TIA-1. To identify which TNFα receptor mediates cardio-protection by TNFα, we also studied mouse hearts that do not express TNFα receptor 1 (TNFR1).

TIA-1 is a RNA-binding protein that binds to the adenine- and uracil-rich element (ARE) in the 3′ untranslated region of mRNAs and decreases the translational efficiency of the associated mRNA transcripts [Piecyk et al., 2000; Lopez de Silanes et al., 2005]. Peritoneal macrophages isolated from mice lacking the gene for

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TIA-1 (TIA-$1^{-/-}$) upon LPS stimulation over-express TNFα protein by ~3-fold relative to WT macrophages [Piecyk et al., 2000], but do not constitutively accumulate higher levels of TNFα mRNA. We therefore hypothesized, and here we show, that the TIA-$1^{-/-}$ mice could be used as a means of acutely over-expressing TNFα protein in mouse hearts during an experimental protocol. We found that both TIA-$1^{-/-}$ and hTNFα$^{+/+}$ hearts have low over-expression of TNFα that is associated with improved cardiac performance, likely via a TNFR1-mediated mechanism.

MATERIALS AND METHODS

ANIMALS

Four groups of 10–16-week-old C57BL/6 male mice were used: 16 mutant mice lacking TIA-1 backcrossed into C57BL/6 mice for 5 generations (TIA-$1^{-/-}$) [Piecyk et al., 2000]; 5 mice expressing the human TNFα transgene (hTNFα$^{+/+}$, from Taconic Farms, Inc.); 10 mutant mice lacking TNFα receptor-1 (TNFR$1^{-/-}$, from Jackson Laboratories) and 25 age-matched non-transgenic mice (WT from Taconic Farms, Inc. and Jackson Laboratories). Body weights, heart weights, left ventricular (LV) volumes and the ratios of heart to body weights and LV volumes to heart weights (both ratios are measures of cardiac hypertrophy) for TIA-$1^{-/-}$, hTNFα$^{+/+}$, TNFR$1^{-/-}$, and WT mice were not significantly different (Table I). The experimental protocols for this study were approved by the Standing Committee on Animals of Harvard Medical Area and followed the recommendations of current NIH and American Physiological Society guidelines for the use and care of laboratory animals.

ISOLATED PERFUSED MOUSE HEART PREPARATION AND MEASUREMENT OF ISOVOLUMIC CONTRACTILE PERFORMANCE

Mouse hearts were isolated and perfused in the Langendorff mode (balloon-in-LV) with a modified Krebs-Henseleit buffer (in mM: 118 NaCl, 5.3 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 0.5 EDTA, 25 $NaHCO_3$, 0.5 pyruvate, and 10 glucose) [Chu et al., 1996; Saupe et al., 1998]. Contractile performance data were collected on-line at a sampling rate of 400 Hz using a digital data acquisition system (MacLab ADInstruments, Milford, MA). LV developed pressure (LV DevP, the difference between systolic pressure (SP) and end diastolic pressure (EDP)), the minimum and maximum values of the first derivative of LV pressure (+dP/dt and −dP/dt), and rate-pressure product (RPP, product of DevP and heart rate (HR)) were calculated off-line.

EXPERIMENTAL PROTOCOLS

Four protocols were used. In the first, 6 pairs of WT and TIA-$1^{-/-}$ hearts were perfused for 2, 30, or 60 min and then rapidly frozen in RNA later solution (Ambion) for measurement of TNFα mRNA and protein levels. In the second protocol 9 WT, 5 TIA-$1^{-/-}$, and 4 TNFR$1^{-/-}$ hearts underwent 30 min of perfusion followed by 24 min of global no-flow ischemia followed by 50 min of reperfusion. During this protocol ^{31}P NMR spectra were obtained. In the third protocol, insulin-independent glucose uptake in 3 WT and 4 TIA-$1^{-/-}$ hearts was measured during the recovery period following 24 min global no-flow ischemia. After 8 min of perfusion with a phosphate-containing Krebs-Henseleit buffer, the buffer was switched to a buffer containing the glucose analog 2-deoxyglucose (2 mM, 2DG) instead of glucose [Abel et al., 1999]. The rate of accumulation of 2DG phosphate (2DGP) measured by ^{31}P NMR spectroscopy closely approximates the rate of 2DG entry into the myocyte, because 2DGP is slowly metabolized. ATP production in the hearts was maintained by supplying 5 mM pyruvate in the perfusate. In the fourth protocol, the ability of the heart to increase isovolumic work in response to increased extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$) was determined using 13 WT, 9 TIA-$1^{-/-}$, 5 hTNFα$^{+/+}$, and 5 TNFR$1^{-/-}$ hearts. After measuring baseline cardiac performance at 2.5 mM Ca^{2+}, the buffer was switched to one containing 1.5 mM Ca^{2+}. After 4 min $[Ca^{2+}]_o$ was increased to 2.0 mM by adding 0.5 mM Ca^{2+} from a 50 mM stock solution at 1% of coronary flow. Then the buffer was switched back to the one containing 2.5 mM Ca^{2+}. $[Ca^{2+}]_o$ was then increased stepwise in 0.5 mM increments to 4.0 mM Ca^{2+}. At each $[Ca^{2+}]_o$ cardiac performance was measured after reaching steady state conditions (5 min). At the end of each protocol hearts were either weighed and stored at −80°C or were kept in RNAlater solution (Ambion) for determination of TNFα mRNA and protein levels.

^{31}P NMR SPECTROSCOPY

^{31}P NMR free induction decays (FIDs) were acquired at 161.8 MHz using a Varian Inova NMR spectrometer (Varian NMR Systems, Palo Alto, CA). For each spectrum, 104 FIDs were averaged using a 60° pulse and a recycle time of 2.5 s for an overall acquisition time of 4 min. The FIDs were Fourier transformed, phased, baseline corrected, and line broadened by 20 Hz. Resonance areas and chemical shifts were quantified using the MacNuts-Utility transform software (Acorn NMR Inc., Livermore, CA). See Saupe et al. 1998 for details on calculating metabolite concentrations and pH.

TNFα mRNA AND PROTEIN MEASUREMENTS

Hearts were stored in 1 ml RNAlater (Ambion) at −20°C. Pairs of WT and TIA-$1^{-/-}$ hearts were mechanically homogenized in 2 ml ice-cold lysis buffer (in mM: 20 Tris pH 7.8, 150 NaCl, 10 EDTA, 2 DTT; and 0.75 U/μl Superasin (Ambion), 5 μm benzamidine, 10 μM PMSF, 5 nM leupeptin, and 5 ng/ml aprotinin). Total RNA was isolated from 0.5 ml of the lysate using Trizol LS (Invitrogen)

TABLE I. Body Weights, Heart Weights, and Left Ventricular Volumes

	n	BW (g)	HW (mg)	HW/BW (mg/g)	LV vol (μl)	LV vol/HW (μl/mg)
WT	25	23.9 ± 1.1	107.7 ± 4.7	4.5 ± 0.1	17.5 ± 0.8	0.17 ± 0.01
TIA-$1^{-/-}$	16	24.9 ± 1.1	111.9 ± 4.6	4.5 ± 0.1	18.4 ± 1.2	0.17 ± 0.02
hTNFα$^{+/+}$	5	28.6 ± 1.8	124.1 ± 9.2	4.3 ± 0.2	22.3 ± 0.5	0.18 ± 0.01
TNFR$1^{-/-}$	10	22.9 ± 0.9	105.9 ± 2.5	4.6 ± 0.1	16 ± 0.5	0.16 ± 0.01

Background of all mouse models is C57BL/6. WT, Wildtype; TIA-$1^{-/-}$, mice lacking TIA-1; hTNFα$^{+/+}$, mice over-expressing human TNFα; TNFR$1^{-/-}$, mice lacking TNF receptor 1; BW, body weight; HW, heart weight; LV vol, left ventricular volume. Means ± SE.

according to the manufacture's protocol. Ten micrograms of each RNA sample was loaded into a lane of a denaturing 1.3% agarose gel. Following electrophoresis and transfer to Nytran, TNFα mRNA levels were determined by hybridization with a ^{32}P-labeled random-primed probe and autoradiography. The remaining 1.5 ml of tissue lysate was used for analysis of TNFα protein levels. NP-40 and SDS were added to final concentrations of 1% and 0.1%, respectively. After pre-clearing with 20 μl of protein A/G sepharose, 4 μg of polyclonal anti-TNFα antibodies (Santa Cruz Biotechnology, Inc.) and 20 μl protein A/G sepharose were added. Half of each immunoprecipitate was resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed for TNFα protein levels by immunoblotting with a combination of two monoclonal antibodies (Pharmingen).

WESTERN BLOTTING FOR SERCA2a AND CALMODULIN

Protein extracts were obtained from LV tissue in HNTG lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10% Glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1.0 mM EDTA, Protease Inhibitor Cocktail, 200 μM Sodium orthovanadate, 1 mM NaF, 5 mM β-glycerolphosphate). Protein concentration was determined according to Lowry [Lowry et al., 1951]. Equal protein amounts were loaded onto 8% (for SERCA2a) or 15% (for calmodulin) SDS-PAGE. Proteins were transferred to nitrocellulose membranes, which were probed with protein specific antibodies (SERCA: Sigma, Saint Louis, MO; calmodulin: Epitomics, Burlingame, CA). Bands were visualized using HRP linked secondary antibodies. GAPDH was used as a loading control.

STATISTICAL ANALYSIS

All results are expressed as mean ± SE. Repeated measures were tested for statistical differences with ANOVA repeated measures and Student–Newman–Keul's post hoc comparison. When appropriate, factorial ANOVA was used to test paired comparisons. Analysis were performed with STATVIEW (Brain Power, Calabasas, CA) or GraphPad Prism 4.0 (Graph Pad Software Inc., San Diego, CA) and a value of $P < 0.05$ was considered significant.

RESULTS

TNFα mRNA AND PROTEIN LEVELS IN PERFUSED WT, TIA-1$^{-/-}$, AND hTNFα$^{+/+}$ HEARTS

High TNFα concentrations (~10^{-9} M) have been associated with decreased inotropy, increased apoptosis, and lower levels (10^{-11} M) with protection from ischemia-reperfusion injury. Here, to test whether these disparate effects are concentration-dependent, we used perfused hearts isolated from two genetically modified mouse models.

To define the expression of TNFα in TIA-1$^{-/-}$ hearts under these conditions, we measured TNFα mRNA and protein levels in WT and TIA-1$^{-/-}$ hearts perfused for 2, 30, or 60 min (Fig. 1A, lanes a). After 2 min of perfusion, neither TNFα mRNA nor protein was detected in either WT or TIA-1$^{-/-}$ hearts. After 30 min perfusion, TNFα mRNA was induced to equal levels in both WT and TIA$^{-/-}$ mice; however, two- to threefold more TNFα protein accumulated in TIA-1$^{-/-}$ hearts. After 60 min perfusion, the differential induction of TNFα protein between WT and TIA$^{-/-}$ hearts was more pronounced. These results demonstrate that (1) isolating and perfusing mouse hearts is sufficient to increase TNFα expression in normal mouse hearts, (2) the increase is rapid, occurring within 30 min, and (3) more TNFα protein is expressed in TIA1$^{-/-}$ compared to WT hearts from comparable amounts of TNFα transcripts. Hearts over-expressing the human TNFα transgene also accumulate two- to threefold more TNFα protein than WT hearts (Fig. 1A, lanes b). Thus, relatively low over-expression of TNFα occurs in both hTNFα$^{+/+}$ hearts (chronic over-expression) and TIA-1$^{-/-}$ hearts (acute perfusion-induced over-expression), making them suitable models for these studies.

CONTRACTILE PERFORMANCE DURING ISCHEMIA AND REPERFUSION

The contractile performance of hearts isolated from TIA-1$^{-/-}$, TNFR1$^{-/-}$ and WT mice during and after an ischemic insult was examined by subjecting these hearts to 24 min of zero-flow ischemia followed by 50 min of reperfusion. This stress is severe: hearts stop beating within seconds of zero-flow perfusion, diastolic stiffness develops as evidenced by increased EDP, and recovery of systolic performance is typically only ~50% with persistent diastolic impairment.

Figure 2 shows average values for indices of systolic and diastolic performance for the three groups of hearts. During 24 min of global no-flow ischemia, all hearts ceased beating (RPP is zero, Fig. 2e,f) and EDP slowly increased (Fig. 2a,b). Although the increase in EDP by 24 min of ischemia was similar in all groups, the onset and the rate of rise of EDP differed among the groups. TIA-1$^{-/-}$ hearts had the slowest increase in EDP whereas hearts without TNFR1$^{-/-}$ showed the fastest and earliest increase of EDP.

During reperfusion, recovery of diastolic performance (assessed as a fall in EDP and an increase in the rate of relaxation, −dP/dt) paralleled the differences in diastolic dysfunction observed during ischemia among hearts. TIA-1$^{-/-}$ hearts recovered better than WT hearts, whereas TNFR1$^{-/-}$ hearts recovered worse. EDP of TIA-1$^{-/-}$ hearts was lower than for WT controls during early (but not late) recovery (Fig. 2b), and −dP/dt was substantially better than for WT throughout reperfusion and recovered to near-normal levels (77% for TIA-1$^{-/-}$ vs. 61% for WT (Fig. 2c)). For TNFR1$^{-/-}$ hearts, EDP tracked WT hearts (Fig. 2b) but −dP/dt (Fig. 2c) was severely blunted during early recovery and recovered to only 40% of baseline after 50 min of reperfusion.

Recovery of systolic performance during reperfusion followed the same pattern, with TIA-1$^{-/-}$ hearts showing better recovery compared to WT while TNFR1$^{-/-}$ hearts did not recover as well as WT hearts. Systolic pressure was the same in all groups (data not shown), but, because of the persistent differences in EDP, DevP recovered to 70% in TIA-1$^{-/-}$, 59% in WT and 40% in TNFR1$^{-/-}$ hearts. Similarly +dP/dt (Fig. 2d) recovered to 88% in TIA-1$^{-/-}$, 59% in WT and 43% in TNFR1$^{-/-}$ hearts. The same pattern of recovery was observed for RPP (Fig. 2f). Note that TNFR1$^{-/-}$ hearts started beating 6–10 min later than WT hearts, and that their RPP, which takes into account HR, SP and EDP, was ~15% lower compared to WT hearts.

Thus, in comparison to WT hearts, TIA-1$^{-/-}$ hearts over-expressing TNFα prior to and during (Fig. 1A, lanes a,c) the onset of

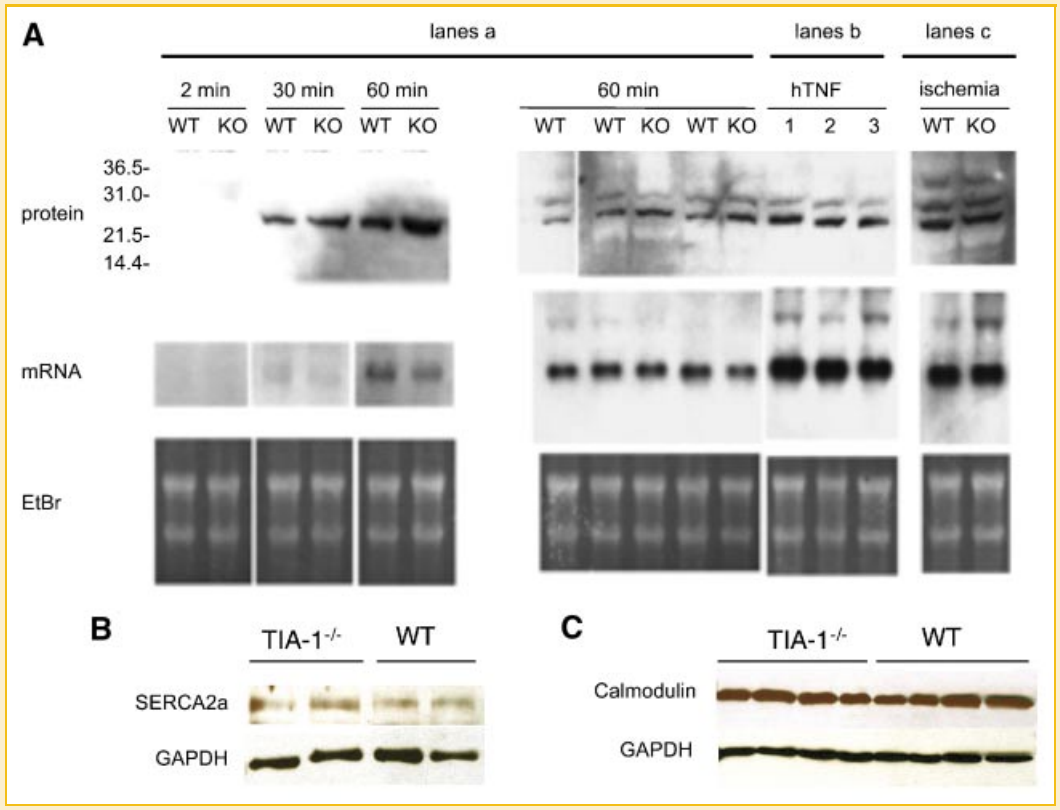

Fig. 1. Representative Northern and Western Blots. Panel A: Northern and Western Blots of TNFα mRNA and protein of WT and TIA-1$^{-/-}$ hearts after 2, 30, and 60 min of Langendorff perfusion (lanes a, left), and of WT, TIA-1$^{-/-}$, and hTNFα$^{+/+}$ hearts after 60 min perfusion with Ca^{2+} challenge (lanes a, right). No TNFα mRNA or protein was detectable after 2 min of perfusion in either group. After 30 and 60 min of perfusion, mRNA levels are similar in both groups, but TIA-1$^{-/-}$ hearts accumulated two- to threefold more protein than WT hearts (lanes a, left). The Ca^{2+} challenge protocol resulted in the same two- to threefold increase of TNFα in TIA-1$^{-/-}$ (lanes a, right). hTNFα$^{+/+}$ hearts also had two- to threefold fold higher TNFα content compared to WT (lanes b). TNFα remained elevated in ischemia (lanes c). Panels B,C show that the expression of SERCA2a and calmodulin is the same in TIA-1$^{-/-}$ and WT hearts. All experiments were repeated at least 2 times with the same results and equal amounts of protein were loaded for each lane.

ischemia perform better during ischemia and recover better during reperfusion, whereas TNFR1$^{-/-}$ hearts exhibit greater diastolic dysfunction during ischemia and recover diastolic function less well during reperfusion. These results suggest that low-dose TNFα has cardio-protective effects during ischemia and reperfusion, and that these effects are mediated through TNFR1. They also suggest that deletion of TNFR1 exacerbates ischemia-induced diastolic dysfunction.

To test whether the improved recovery in TIA-1$^{-/-}$ hearts is due to higher levels of ATP and the primary energy reserve compound phosphocreatine (PCr), we used ^{31}P NMR spectroscopy to measure their concentrations throughout the ischemia-reperfusion protocol. No differences were observed (Table II) between the mouse hearts. Because increasing glucose supply to the ischemic heart has been shown to lead to improved performance [Van Rooyen et al., 2002], and TNFα is known to increase glucose uptake via increased insulin-independent glucose transporter (GLUT-1) in skeletal muscle [Evans and Eisenberg, 1989], we then tested whether insulin-independent glucose uptake rate (assessed as 2-DG uptake rate) was higher in TIA-1$^{-/-}$ hearts during reperfusion. TIA-1$^{-/-}$ hearts have 40% lower, not higher, uptake of glucose after ischemia (Fig. 3). These data indicate that the increased contractile performance in TIA-1$^{-/-}$ hearts is not due to improved energetics assessed as glucose uptake and intracellular ATP levels. These findings suggest that TIA-1$^{-/-}$ have improved cardiac efficiency.

The mRNAs for SERCA2a and calmodulin have a binding motif for TIA-1 and can be immunoprecipitated complexed to TIA-1 with anti-TIA-1 antibodies [Lopez de Silanes et al., 2005]. To determine whether the loss of TIA-1 in our TIA-1$^{-/-}$ mouse model changes the protein levels of SERCA2a and calmodulin, and thus could influence contractile performance, we performed Western blots for WT and TIA-1$^{-/-}$ hearts. Figure 1B,C shows that SERCA2a and calmodulin protein levels are the same for both groups of mice.

CONTRACTILE PERFORMANCE IN RESPONSE TO VARYING $[Ca^{2+}]_o$

To test whether mouse hearts with low over-expression of TNFα demonstrate improved contractile performance by increasing Ca^{2+} sensitivity, we then measured systolic and diastolic contractile performance in hearts over-expressing either murine or human TNFα by varying $[Ca^{2+}]_o$. Figure 4 shows that, over a wide range of $[Ca^{2+}]_o$, all indices of systolic (DevP panel a, RPP panel b, and +dP/dt panel d) and diastolic (−dP/dt panel c) performance measured were higher in TIA-1$^{-/-}$ and hTNF$^{+/+}$α than for WT hearts. The regression lines obtained for TIA-1$^{-/-}$ and hearts are shifted to the

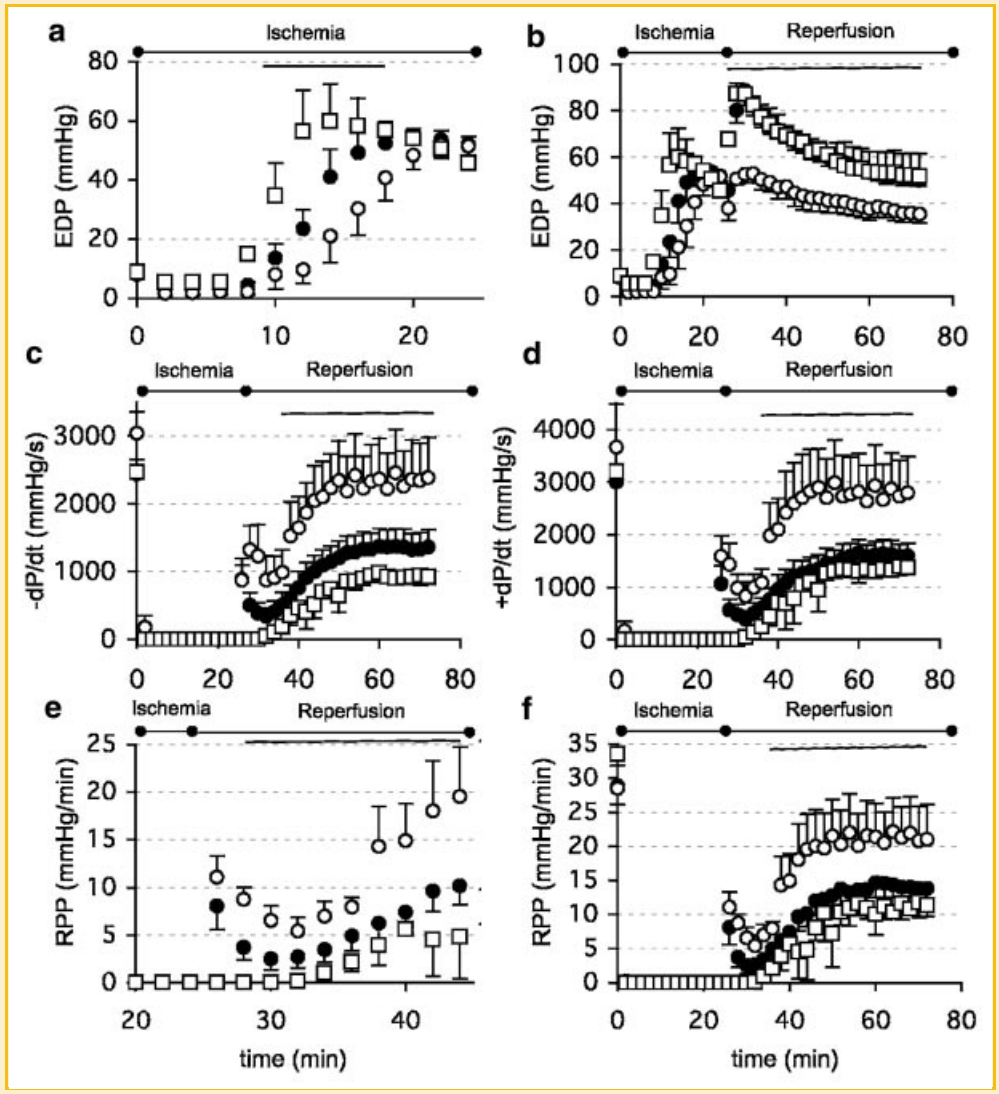

Fig. 2. Isovolumic contractile performance of WT (filled circles, n = 10), TIA-1$^{-/-}$ (open circles, n = 5) and TNFR1$^{-/-}$ (open squares, n = 5) hearts at baseline, during 24 min of no-flow ischemia and 50 min reperfusion. Compared to WT hearts, the increase in EDP characteristic of no-flow ischemia occurred earlier in TNFR1$^{-/-}$ hearts and later in TIA-1$^{-/-}$ hearts (panels a,b). TIA-1$^{-/-}$ hearts show better recovery from ischemia compared to WT hearts for all indices of systolic and diastolic performance (panels a–f). In contrast, TNFR1$^{-/-}$ hearts show poorer recovery: diastolic performance measured as EDP (panels a,b) and −dP/dt (panel c) and the early transient increase in systolic performance assessed as +dP/dt (panel d) and RPP (panel f) is absent. These results suggest that low over-expression of TNFα leads to improved contractile performance during and following ischemia and that the absence of TNFR1 impairs the ability of hearts to tolerate acute ischemic stress. Means ± SE, bold horizontal lines indicate data with $P < 0.05$ ANOVA repeated measures, Student–Newman–Keul's post hoc comparison versus WT.

TABLE II. Concentrations of Inorganic Phosphate, Phosphocreatine, ATP, and pH at Baseline and Steady State Reperfusion After 24 min Global No-Flow Ischemia in WT and TIA-1$^{-/-}$ Hearts

	WT		TIA-1$^{-/-}$	
	Baseline	Reperfusion	Baseline	Reperfusion
Pi	3.1 ± 1.1	20.7 ± 2.8	2.5 ± 0.8	19.4 ± 2.9
PCr	17.6 ± 0.7	12.5 ± 0.9	19.2 ± 0.3	14.8 ± 1.8
ATP	9.6 ± 0.3	2.5 ± 0.4	9.9 ± 0.3	3.5 ± 0.6
pH	7.14 ± 0.05	7.00 ± 0.02	7.19 ± 0.01	7.04 ± 0.04

WT, Wildtype; TIA-1$^{-/-}$, mice lacking TIA-1; inorganic phosphate, Pi; PCr, phosphocreatine. Means ± SE.

left by on average of 0.20 and 0.26 mM pCa, respectively. Table III shows results obtained at 1.5 mM $[Ca^{2+}]_o$. These results suggest that low over-expression of TNFα improves cardiac contractile performance of the isolated heart.

CONTRACTILE PERFORMANCE IN TNFR1$^{-/-}$ MOUSE HEARTS

To determine which TNF receptor (TNFR1 or TNFR2) mediates improved contractile performance, we repeated the Ca^{2+} challenge protocol using TNFR1$^{-/-}$ mouse hearts. hTNF$^{+/+}$α binds primarily to murine TNFR1, which has similar affinities for human and murine TNFα, in contrast to murine TNFR2 which has strong specificity for murine TNFα [Lewis et al., 1991]. Results in Figure 5 demonstrate that deletion of TNFR1 abolishes increased Ca^{2+} sensitivity. Taken

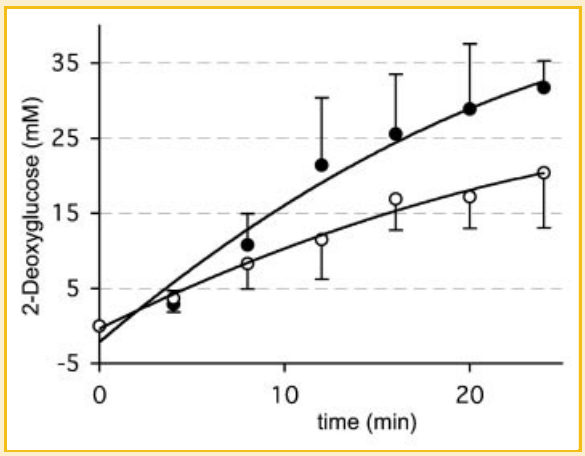

Fig. 3. 2-Deoxyglucose uptake of TIA-$1^{-/-}$ (open circles) and WT (filled circles) hearts after ischemia. TIA-$1^{-/-}$ exhibit a blunted glucose uptake rate compared to WT despite similar cardiac ATP and PCr content but improved contractile performance. Means ± SE, n = 4, bold horizontal line indicates data with $P < 0.05$ ANOVA repeated measures, Student–Newman–Keul's post hoc comparison versus WT.

TABLE III. Indices of Isovolumic Contractile Performance of Hearts Over-Expressing TNFα at $[Ca^{2+}]_o$ of 1.5 mM

	WT	TIA-$1^{-/-}$	hTNFα$^{+/+}$
DevP (mmHg)	23.5 ± 2.2	37.6 ± 2.8*	47.6 ± 9.5*
RPP (×10^3 mmHg/min)	6.7 ± 1.1	12.5 ± 1.4*	18.7 ± 3.1*
+dP/dt (mmHg/s)	1,036 ± 108	1,649 ± 170*	1,667 ± 210*
−dP/dt (mmHg/s)	435 ± 41	756 ± 86*	957 ± 219*

WT, Wildtype; TIA-$1^{-/-}$, mice lacking TIA-1; hTNFα$^{+/+}$, mice over-expressing human TNFα; developed pressure (systolic pressure minus end diastolic pressure), DevP; rate pressure product (product of heart rate and devP), RPP; rate of tension development, +dP/dt; rate of relaxation, −dP/dt.
Means ± SE.
*$P < 0.05$ versus WT by ANOVA.

together, these results suggest that the improved contractile performance observed in hearts over-expressing TNFα is mediated by TNFR1.

DISCUSSION

The cytokine TNFα has pleiotropic functions in various tissues ranging from mediating apoptotic responses to inducing survival pathways [Kapadia et al., 1995b; Chen and Goeddel, 2002; MacEwan, 2002; Hunter et al., 2003; Haudek et al., 2007]. In the heart, TNFα has been associated with mediating negative inotropic

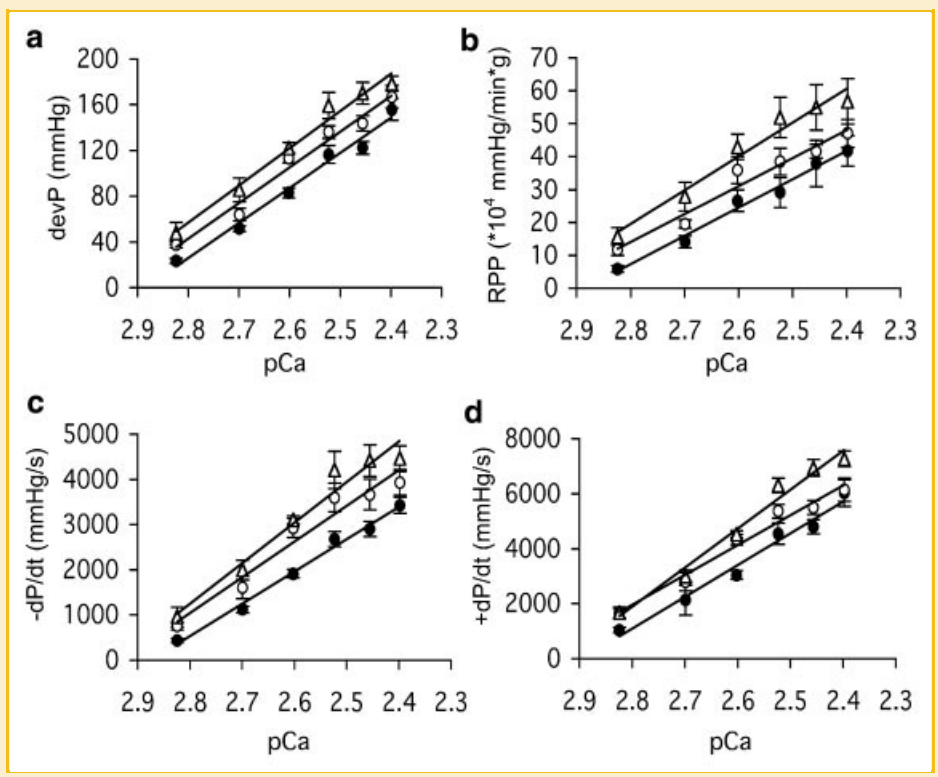

Fig. 4. Indices of systolic (DevP, panel a; RPP/g LV, panel b; and +dP/dt, panel c) and diastolic (−dP/dt, panel d) performance for WT (filled circles), TIA-$1^{-/-}$ (open circles) and hTNFα$^{+/+}$ hearts (open triangles) vs. increasing $[Ca^{2+}]_o$ expressed as pCa. At any $[Ca^{2+}]_o$, TIA-$1^{-/-}$ and hTNFα$^{+/+}$ hearts have improved systolic and diastolic performance compared to WT hearts. This is the case even if the small differences in heart weights are taken into account by normalizing RPP by heart weight (panel b). The regression lines for TIA-$1^{-/-}$ and hTNFα$^{+/+}$ hearts are shifted to the left by on average of 0.2 and 0.26 mM pCa, respectively. Data are expressed as means ± SE. Linear regression analysis performed using GraphPad Prism, *y*-axis intercepts are different with $P < 0.01$.

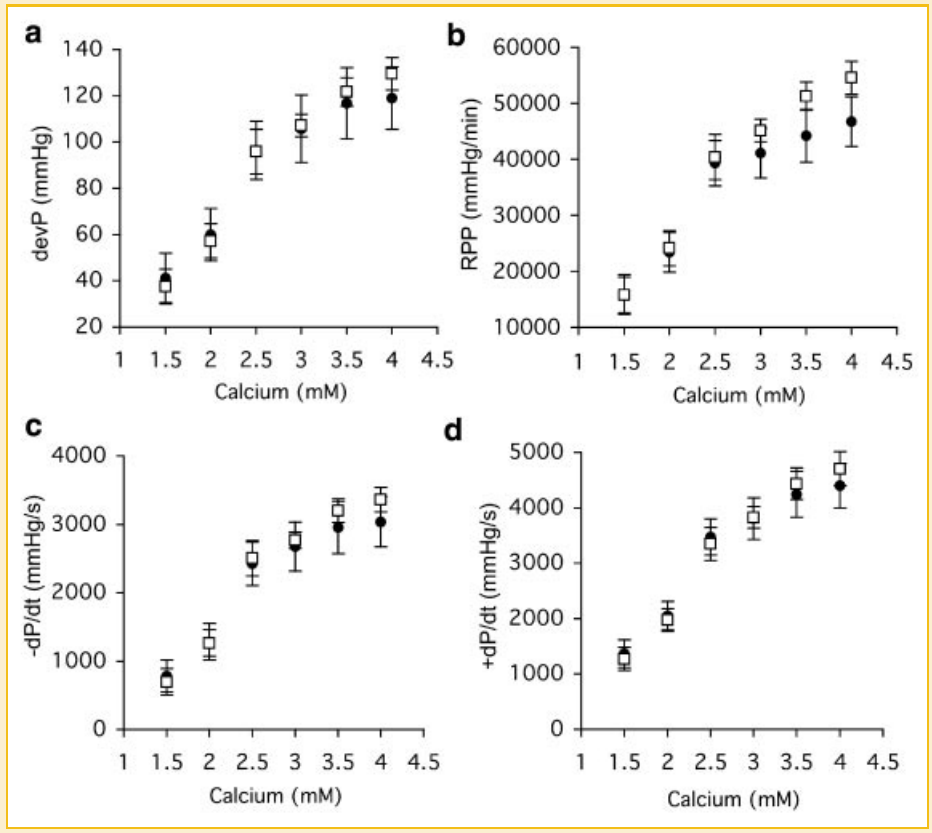

Fig. 5. Indices of systolic (DevP, panel a; RPP, panel b; and +dP/dt, panel c) and diastolic (−dP/dt, panel d) performance of WT (filled circles) and TNFR1$^{-/-}$ (open squares) hearts at different $[Ca^{2+}]_o$. There are no differences in any of the indices of contractile performance, suggesting TNFR1 is not necessary for the WT level of contractile performance. Means ± SE, n = 5 both groups.

effects, thereby contributing to the progression of heart failure. Experimental over-expression of TNFα in the mouse heart leads to rapid induction of dilatation in only 4–12 weeks [Sivasubramanian et al., 2001]. TNFα has also been reported, however, to activate survival pathways in several tissue types, including bone, pancreas, nerve, and smooth muscle. In the heart, Lecour et al. 2002 found that $\sim 10^{-11}$M of soluble TNFα protected cardiomyocytes from ischemia/reperfusion injury. It is not clear how TNFα can induce both beneficial and detrimental signaling pathways. It is possible that the amount of TNFα expression determines which pathway is activated (or dominant) and which molecular targets are affected. To test this, we compared indices of contractile performance in two mouse hearts that express low amounts (two- to threefold) of TNFα protein (hTNFα$^{+/+}$, TIA-1$^{-/-}$) and mouse hearts without the TNF receptor 1 (TNFR1$^{-/-}$).

LOSS OF TIA-1 CAUSES LOW OVER-EXPRESSION OF TNFα IN ISOLATED HEARTS

TNFα mRNA and protein are rapidly induced (by 30 min) in hearts isolated from both WT and TIA-1$^{-/-}$ mice, but TIA-1$^{-/-}$ hearts accumulated more TNFα protein from similar levels of RNA transcripts. The procedure used to remove the heart and attach it to the perfusion apparatus involves many stresses (mechanical and metabolic) that may contribute to the induction of TNFα production: (1) temperature stress: hearts are arrested in ice cold buffer prior to perfusion, (2) hypoxia: prior to attachment for about 2 min due to no perfusion, (3) mechanical: perfused with 10 times higher coronary flow rates than in vivo, and (4) loss of neural and hormonal input. This experiment cannot distinguish which stress or combination of stresses induces TNFα expression. For the purposes of this study it was sufficient to induce TNFα expression by the isolation procedure of the heart. The loss of TIA-1 causes two- to threefold over-expression of TNFα protein in the perfused heart. This is approximately the level of TNFα protein observed in LPS-stimulated TIA-1$^{-/-}$ macrophages [Piecyk et al., 2000].

LOW TNFα PROTECTS HEARTS FROM ISCHEMIC INJURY

The low over-expression of TNFα in TIA-1$^{-/-}$ hearts prior to and during the onset of ischemia was associated with cardio-protection during the ischemia/reperfusion protocol. These hearts recovered systolic and diastolic function better compared to WT hearts. Our results are supported by the findings of Rathi et al. 2002 and Lecour et al. 2002. In our experiments, however, TNFα was present throughout the protocol, whereas Lecour et al. 2002 found a protective effect only after washing out TNFα. This difference could be due to different metabolic effects specific to membrane bound TNFα, which is present during the experimental conditions of this

study but not that of Lecour et al. 2002 where TNFα was supplied in the perfusate.

In order to determine which of the two TNF receptors mediates the protection from ischemic damage, the ischemia experiments were repeated with TNFR1$^{-/-}$ hearts. We found that deletion of TNFR1 abolishes the improvement in contractile performance observed in hearts with low TNFα. Furthermore, hearts without TNFR1 demonstrated worse diastolic function during ischemia and early during reperfusion. The results suggest two conclusions: first, the protective effect of TNFα during reperfusion is mediated by TNFR1, and second, the initial blunted recovery of TNFR1$^{-/-}$ hearts during early reperfusion suggests that TNFα may be required for the rapid recovery of ischemic tissue. The diminished contractile performance in TNFR1$^{-/-}$ hearts compared to WT hearts during early reperfusion observed here is in contrast to the findings of Kurrelmeyer et al. 2000 studying TNFR1$^{-/-}$ hearts 24 h after regional ischemia. In that study TNFR1$^{-/-}$ hearts had similar infarct size as WT hearts, which suggest similar contractile performance. Differences in timing (early reperfusion vs. 24 h after regional infarction) and amount of TNFα (greater in the global ischemia model studied here) in these two protocols likely explain the different results.

POSSIBLE MECHANISMS UNDERLYING THE PROTECTIVE ROLE OF LOW LEVELS OF TNFα

To test whether improved contractile performance in the TIA-1$^{-/-}$ hearts was due to increased glycolysis [Van Rooyen et al., 2002] we performed two experiments. First, glucose utilization known to occur during ischemia and early reperfusion should improve cardiac energetics [Cave et al., 2000], we measured [ATP] and [PCr] during ischemia and reperfusion. We did not find differences between the groups. Second, to directly test whether the uptake of glucose differed in reperfused TIA-1$^{-/-}$ hearts, we measured insulin-independent 2-DG uptake early during reperfusion. Increased TNFα has been shown to increase insulin-independent glucose uptake in skeletal muscle [Evans and Eisenberg, 1989]. Unexpectedly, TIA-1$^{-/-}$ had a 40% slower, not faster, insulin-independent glucose uptake rate. This result suggests that two- to threefold increase in TNFα in the heart was sufficient to decrease, not increase, glucose uptake. In obesity it is known that the release of TNFα by adipocytes inhibits insulin-stimulated glucose uptake, leading progressively towards insulin resistance and type 2 diabetes [Hotamisligil, 2003]. Our results suggest that TNFα targets both insulin-independent and -dependent glucose uptake, and that this may be one of the mechanisms of diminished glucose utilization in heart failure.

To determine whether the improved contractile performance in TIA-1$^{-/-}$ hearts is caused by increased Ca^{2+} sensitivity, we measured contractile performance over a wide range of $[Ca^{2+}]_o$. Over the entire range, TIA-1$^{-/-}$ hearts have increased Ca^{2+} sensitivity and all contractile performance parameters (diastolic and systolic) measured improved. It is important to point out that despite the presence of the binding motif for TIA-1 in mRNAs for SERCA2a and calmodulin [Lopez de Silanes et al., 2005], the amount of SERCA2a and calmodulin protein was not changed in TIA-1$^{-/-}$ mouse hearts. Thus, the increase in calcium sensitivity observed here for TIA-1$^{-/-}$ hearts cannot be explained by either increased SR calcium loading or activation of the calmodulin kinase pathway.

To support our conclusion that TNFα is responsible for increased Ca^{2+} sensitivity, Ca^{2+} sensitivity of TNFα over-expressing mice was determined. Both TNFα over-expressors and TIA-1$^{-/-}$ hearts with perfusion-induced TNFα over-expression show increased Ca^{2+} sensitivity and thus improved contractile performance. From Figure 4 and Table III it is also possible to conclude that when even low-levels of TNFα are present throughout the life of the mouse, hearts perform better. These results support the conclusion that both time and amount of TNFα over-expression determine TNFα's effects on the heart.

INCREASED CONTRACTILE PERFORMANCE IS MEDIATED BY TNFR1

Human TNFα binds only to the mouse TNFα receptor TNFR1 and not to TNFR2 [Lewis et al., 1991]; therefore, the increased Ca sensitivity in hTNFα$^{+/+}$ over-expressing hearts suggests that their improved contractile performance is mediated via TNFR1. Depending on the tissue, binding of TNFα to TNFR1 activates pathways that mediate increased Ca^{2+} sensitivity; these include phospholipase A_2, mitogen activated protein kinase (MAPK), rho kinase, c-Jun, and ceramide-sphingosine [Chen and Goeddel, 2002]. In airway smooth muscle cells, for example, TNFα increases Ca^{2+} sensitivity by increasing myosin light chain phosphorylation via either phospholipase A2 or MAPK pathway [Parris et al., 1999] or a rho-kinase dependent pathway [Hunter et al., 2003]. In cultured sensory neurons TNFα causes transient Ca^{2+} waves and activates p38MAPK and c-Jun [Pollock et al., 2002], both stress-related kinases. Furthermore, in the heart, TNFR1 leads to the activation of the ceramide/sphingosine-1-phosphate pathway, which increases intracellular Ca^{2+} release via ryanodine receptors [Scott et al., 2000]. Cardiac ceramide levels and activation of JNK (which is downstream of c-Jun) are not different in WT and TIA-1$^{-/-}$ hearts (unpublished data; Cebova, Knowles, Preda, Vary and Pinz) suggesting another mechanism of increased Ca^{2+} sensitivity in TIA-1 hearts.

It is possible that the beneficial effects of TNFα on cardiac function that we observed in the TIA-1$^{-/-}$ and hTNFα$^{+/+}$ hearts were due to a combination of acute over-expression during the experimental protocol and pre-protocol over-expression of TNFα. It is likely that the transcription of endogenous TNFα is intermittently activated in TIA-1$^{-/-}$ mice under normal conditions. In that case TIA-1$^{-/-}$ mice as well as hTNFα$^{+/+}$ mice (in which the TNFα transgene is under the transcriptional control of the TNFα promoter) would be expected to over-express TNFα protein. Consistent with this notion, TIA-1$^{-/-}$ mice have a greater tendency to develop inflammatory arthritis than WT mice when they age [Phillips et al., 2004].

In conclusion, our results demonstrate that low concentrations of endogenous TNFα protect the heart from ischemia-reperfusion injury and increase Ca^{2+} sensitivity leading to improved contractile performance. Our results provide strong support for the conclusion that the amount of TNFα is a defining factor determining detrimental versus protective effects. These results also have implications for the treatment of patients with ischemic heart disease or heart failure where the goal should perhaps not be complete suppression of TNFα but rather the achievement of an optimal level of TNFα to achieve a fine balance between ''enough and too much.''

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