TNFR1 and TNFR2 Signaling Interplay in Cardiac Myocytes*  

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Tumor necrosis factor α (TNFα) plays a major role in chronic heart failure, signaling through two different receptor subtypes, TNFR1 and TNFR2. Our aim was to further delineate the functional role and signaling pathways related to TNFR1 and TNFR2 in cardiac myocytes. In cardiac myocytes isolated from control rats, TNFα induced ROS production, exerted a dual positive and negative action on [Ca2+] transient and cell fractional shortening, and altered cell survival. Neutralizing anti-TNFα antibodies exacerbated TNFα responses on ROS production and cell death, arguing for a major protective role of the TNFR2 pathway. Treatment with either neutralizing anti-TNFα antibodies or the glutathione precursor, N-acetylcysteine (NAC), favored the emergence of TNFR2 signaling that mediated a positive effect of TNFα on [Ca2+] transient and cell fractional shortening. The positive effect of TNFα relied on TNFR2-dependent activation of the cPLA2 activity, independently of serine 505 phosphorylation of the enzyme. Together with cPLA2 redistribution and AA release, TNFα induced a time-dependent phosphorylation of ERK, MSK1, PKCβ, CaMKII, and phospholamban on the threonine 17 residue. Taken together, our results characterized a TNFR2-dependent signaling and illustrated the close interplay between TNFR1 and TNFR2 pathways in cardiac myocytes. Although apparently predominant, TNFR1-dependent responses were under the yoke of TNFR2, acting as a critical limiting factor. In vivo NAC treatment proved to be a unique tool to selectively neutralize TNFR1-mediated effects of TNFα while releasing TNFR2 pathways.

Tumor necrosis factor α (TNFα) is a potent proinflammatory cytokine produced by many cell types including cardiac myocytes (1). Silenced under normal conditions, myocardial TNFα expression is enhanced upon sustained hemodynamic overloading of the heart or ischemic injury. Levine et al. (2) were the first to correlate circulating levels of TNFα with the severity of chronic heart failure in patients and postulated that TNFα might contribute to the pathogenesis of heart failure. Thereafter, increased circulating TNFα has been shown to be associated with many forms of cardiac injury, including acute viral myocarditis, myocardial infarction, atherosclerosis, chronic heart failure, cardiac allograft rejection, and sepsis-associated cardiac dysfunction (1). These studies clearly highlight that a control of the TNFα destructive role in cardiovascular disease represents a realistic goal for clinical medicine.

Nevertheless, a large body of evidence indicates that TNFα exerts beneficial effects on the heart (3). In fact, high levels of TNFα are detected in patients with well compensated heart failure, suggesting that TNFα may serve as a short term adaptive response and initiate cardiac remodeling (4–7). This protective action of TNFα might explain why large scale, randomized, placebo-controlled trials with TNFα antagonists have failed to show any improvement in the clinical status of heart failure and even highlighted worsening of the clinical condition of patients with moderate to severe heart failure (8, 9).

The biological responses to TNFα are mediated through two structurally distinct receptors: type 1 (TNFR1) and type 2 (TNFR2), both expressed in cardiac myocytes (10). Although the exact functional significance of TNFR1 and TNFR2 in the heart is not known at present, the majority of the deleterious effects of TNFα are related to the activation of TNFR1, and include short term negative inotropic effects (10), and long term TNFα-induced cell death (11). In contrast, activation of TNFR2 appears to exert protective effects (12, 13). Although the cardiac TNFR1 downstream signaling system has been studied extensively (1), the transduction of signals from TNFR2 and its role in TNFα signaling remains far less well characterized.

TNFα has been shown to induce oxidant stress and to cause a drop in glutathione levels, which precedes and regulates its cytotoxic effects (14). Alternatively, in vivo studies, we have previously shown that a glutathione precursor, antioxidant molecule, NAC prevents the deleterious effect of TNFα on cardiac myocyte contraction from control rats, and hinders the progression of cardiac injury in hypertensive L-NAME-treated rats and in post-myocardial infarction rats (15–17).

The present study was undertaken to further delineate the role and the signaling pathways of TNFR1 and TNFR2 in cardiac myocytes. Our working hypothesis was that NAC treatment might be a unique tool to characterize TNFR2-dependent signaling pathways insofar as protective action of NAC against TNFα might rely on TNFR1 signaling inhibition. In this study, we compared TNFα signaling pathways, in cardiac myocytes isolated from control or NAC-treated rats and investigated the...
impact of neutralizing TNFR1 or TNFR2 antibodies. Our results clearly demonstrate that TNFR1 mediates production of ROS, dual positive and negative effects on [Ca^{2+}]_i, handling and cell fractional shortening and cell death. In contrast, TNFR2 plays a major protective role through inhibition of ROS production and cell death. Treatment with either NAC or neutralizing TNFR1 antibodies reveal a new TNFR2-dependent positive effect on [Ca^{2+}]_i handling and cell fractional shortening mediated by activation of cPLA_2, PKC_\varepsilon, and CaMKII pathways, leading to threonine 17 phosphorylation of phospholamban.

**EXPERIMENTAL PROCEDURES**

**Materials**—TNF\(\alpha\) was from R&D Systems. NAC, MAFP, and L-NAMe were from Sigma. The PKC-\(\zeta\) pseudosubstrate was from BIOSOURCE (Clinisciences, Montrouge, France). [5,6,8,9,11,12,14,15-\(^3\)H]Arachidonic acid (180–240 Ci/mmol) was from GE Healthcare (Les Ulis, France).

Rabbit polyclonal antibodies against phospho-p44/p42 (Thr202/Tyr204), against phospho-MSK1 (Thr581) and against phospho-cPLA_2 (Ser286) were from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies against phospho-CaMKII\(\alpha\) (Thr286), clone 22B1 were from VWR. Rabbit polyclonal antibodies against phospho-PKC_\varepsilon (Thr410) and monoclonal antibodies against cPLA_2 were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against phospho-PLB (Thr17) were from Cyclacel (Dundee, UK). Neutralizing monoclonal antibodies against TNFR1 (Mab 225) and TNFR2 (Mab 226) were from R&D. Rabbit polyclonal antibodies against actin were from Sigma. Peroxidase-conjugated goat anti-rabbit IgG (H+L) or goat anti-mouse IgG (H+L) and the chemiluminescent detection kit (Supersignal West Dura) were from Pierce. FITC-conjugated donkey anti-mouse antibodies were from Jackson Laboratories.

**Cardiac Myocyte Isolation**—The care and the use of animals were in accordance with institutional guidelines. Adult, male Wistar rats (180–250 g, Janvier, LeGenest St Isle, France) were used. Rats received, or not, NAC (Sigma) added to the drinking water (50 mg/d per animal), for 2 weeks. Calcium-tolerant Wistar rats (180–250 g, Janvier, LeGenest St Isle, France) were in accordance with institutional guidelines. Adult, male rats were from Jackson Laboratories.

**Measurement of [Ca^{2+}]_i Imaging**—Imaging experiments were performed at room temperature in BSS buffer (in mM: 130 NaCl, 5 KCl, 5 MgCl_2, 2 CaCl_2, 200 glucose, and 50 HEPES, pH 7.4). Cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Molecular Probes) was used to visualize intracellular ROS. Cells were exposed to 5 \(\mu\)M H2DCF-DA and 1.5 \(\mu\)M Fura2-AM, for 20 min at room temperature, to combine ROS and [Ca^{2+}]_i imaging. After washing, cells were checked for appropriate [Ca^{2+}]_i transient responses to electrical stimulation, as well as to TNF\(\alpha\), before and after recording of the DCF fluorescence images, respectively. DCF fluorescence was recorded as previously reported (16), using 480 nm and 540 nm as excitation and emission wavelength, respectively. At the end of each experiment, maximal DCF fluorescence was determined in response to 2.5 \(\mu\)M H2O2. Results are shown as typical representation from experiments performed on at least 10 cells obtained from three different cell isolations.

**Measurement of ROS Production in Isolated Cardiac Myocytes**—Cardiac myocytes (7,000 cells/well in 96-well plates precoated with laminin) were allowed to attach overnight. After one wash with BSS buffer, cells were loaded for 30 min at 37 °C with 5 \(\mu\)M H2DCF-DA together with increasing concentrations of anti-TNFFR1- or anti-TNFR2-Mabs or vehicle, in BSS buffer. After one wash with BSS buffer, either 25 ng/ml TNF\(\alpha\), 2.5 mM H_2O_2, or vehicle were added, and fluorescence at excitation and emission wavelengths of 485 and 530 nm, respectively, was monitored every 5 min for 30 min (FL-600 multiplate fluorimeter, Biotek Instruments). Values were corrected for cell autofluorescence. Results were the mean of three different experiments performed on two different cell isolations.

**Measurement of Cell Survival**—Cardiac myocytes isolated from control rats were preincubated for 30 min with or without 3 \(\mu\)g/ml anti-TNFFR1- or TNFR2-Mabs before addition or not of 25 ng/ml TNF\(\alpha\), and cultured for 18 h. In parallel, cardiac myocytes isolated from NAC-treated rats were cultured for 18 h, in the presence or in the absence of 25 ng/ml TNF\(\alpha\). Cardiac myocytes were visualized using brightfield at \(\times100\) magnification, and survival was estimated by counting viable rod-shaped cells versus contracted, nonrod-shaped, dead cells, in 20 random microscopic fields. At least 300 cells were counted in each dish, and results were the mean of two different experiments performed on two different cell isolations.

**Measurement of \[^{3}\text{H}\]AA Release**—Freshly isolated cardiac myocytes were plated in 12-well plates previously coated with 10 \(\mu\)g of laminin (30,000 cells/well). Following adhesion (4 h), the cell medium was replaced by fresh medium supplemented with \[^{3}\text{H}\]AA (0.3 \(\mu\)Ci/ml) and cells kept in culture for 24 h in humidified 6% CO_2, 95% air at 37 °C. Where indicated, neutralizing TNFR1 or TNFR2 antibodies were added during the last 30 min of incubation. Cells were kept at 37 °C, washed twice with 1 ml of BSS buffer containing 0.2% fatty acid-free bovine serum albumin, and resuspended in 1 ml of the same buffer. At time 0, cells were exposed to TNF\(\alpha\) or vehicle, and medium samples of 200 \(\mu\)l were taken at time 5, 10, and 20 min, transferred to microcentrifuge tubes and diluted with 200 \(\mu\)l of ice-cold EGTA (4 mm final). After centrifugation at 17,600 \(\times\) g for 10 min at 4 °C, the amount of radioactivity in the supernatants was quantitated by liquid scintillation counting, as previously described (16, 20). Results were obtained from quadruplicate determinations. Kinetics analyses of the data showed a linear \[^{3}\text{H}\]AA release during the 20-min period examined, in all conditions tested. This allowed determination of the rate of
[3H]AA release and comparison of rates in the absence and in the presence of TNFα. Results were expressed as TNFα-induced increases in the rate of [3H]AA release and were the mean ± S.E. of three different experiments performed on three different cell isolations.

**Immunoblot Analysis of the Phosphorylation Status of ERK, MSK1, cPLA2, CaMKII, PKCζ, and PLB**—Freshly isolated cardiac myocytes were submitted to pretreatment with or without neutralizing antibodies or MAFF followed by incubation with or without 25 ng/ml TNFα in BSS buffer at 37 °C for the indicated period of time. Following centrifugation, cellular pellets were dissolved in Laemmlí–loading buffer and samples (30 μg) subjected to SDS-PAGE (8% (P-cPLA2), 10% (P-MSK1, P-PKCζ, and P-ERK) and 18% (P-PLB) acrylamide gels). Proteins were electrotransferred to PVDF membranes (0.45 μm (P-ERK, P-MSK1, P-cPLA2, P-CaMKII, P-PKCζ) or 0.22 μm (P-PLB) (Millipore)). Membranes were first incubated with antibodies against P-ERK, P-MSK1, P-cPLA2, P-CaMKII, P-PKCζ or 0.22 μm (P-PLB) (Millipore). Membranes were then treated with secondary FITC-conjugated donkey anti-mouse antibodies (1:2000 dilution of primary antibodies). Images were collected as previously described (18), with a Zeiss LSM 510 multitracking laser scanning confocal microscope (Carl Zeiss SAS, Frankfurt, Germany), laser line 488 nm and an oil objective ×63 (NA 1.4). We studied nine individual myocytes in each condition examined (control versus TNFα, in the presence or absence of anti-TNFRI or TNFRII Mab antibodies). Note that we were only able to analyze TNFα-dependent redistribution of global cPLA2 labeling due to the lack of availability of antibodies directed against P-cPLA2 isoforms other than P-Ser505-cPLA2. Thus, only TNFα-dependent intensification of the cPLA2 signal was detectable without precise enzyme redistribution localization likely due to the limited portion of enzyme redistributed in response to TNFα compared with total cPLA2 labeling.

**Drug Treatments**—Note that a 25 ng/ml TNFα concentration was chosen to favor analysis of the biphasic effect of the cytokine on [Ca2+]i transients and cell fractional shortening (16, 19).

We checked that neutralizing antibodies Mab 225 (anti-TNFRI) and Mab 226 (anti-TNFRII) recognized rat TNFRI (55 kDa) and rat TNFRII (75 kDa) proteins, respectively, on Western blots performed under non reducing conditions. TNFα neutralization was obtained following incubation of cardiac myocytes for 30 min with 3 μg/ml of each antibody, before addition of TNFα. MAFF (4 μg/ml), L-NAME (1 mM), or PS-PKCζ (2.5 μM), were added 10 min before addition of TNFα.

**Myocardial TNFα, TNFRI, and TNFRII Protein Expression Levels**—Rat hearts were rapidly frozen in isopentane cooled with liquid nitrogen, and stored at −80 °C. Left ventricles (LV) were cut into 20-μm sections. Homogenates were prepared from five frozen sections of each LV by homogenization at 4 °C, in 200 μl of 50 mM Hepes, pH 7.4, containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin), using disposable pette/microtube devices (Fisher Scientific). After centrifugation at 4 °C at 20,000 × g for 20 min, sTNF-α, sTNFRI, and sTNFRII were quantified in the supernatant. The pellet, containing the membrane fraction, was resuspended by homogenization in the Hepes buffer containing protease inhibitors, and 1% Triton X-100. After 30 min of incubation on ice, the suspension was centrifuged at 20,000 × g for 20 min, and solubilized membrane-bound TNF-α, TNFRI, and TNFRII in the supernatant were determined with double sandwich ELISA kits from R&D Systems (rat TNFα Quantikine, mouse sTNFRI, and mouse sTNFRII, respectively).

**Statistical Analysis**—Results were analyzed by the unpaired two-tailed Mann-Whitney test using GraphPad Prism 4 software. Differences were considered statistically significant at a value of \( p < 0.05 \).

**RESULTS**

**Respective Role of TNFRI and TNFRII on TNFα Effects on ROS Production, [Ca2+]i Transients and Cell Fractional Shortening and Cell Death**—The amplitude of [Ca2+]i transients was measured in electrically stimulated adult rat cardiac myocytes, loaded with Fura 2-AM, alone or in combination with the ROS-sensitive fluorescent indicator, H2DCF-DA, in response to TNFα. TNFα (25 ng/ml) exerted a dual, early transient positive and late persistent negative effect on [Ca2+]i transients, with a 85 ± 42% increase over basal after 10 min followed by a 46 ± 13 decrease below basal after 30 min of TNFα perfusion (Fig. 1, C and A). Concurrently, as shown in a typical experiment performed in cardiac myocytes coloaded with Fura 2-AM and H2DCF-DA, TNFα elicited an early and sustained ROS production (Fig. 1A). Both early positive and late negative actions of TNFα on [Ca2+]i, transient amplitude were associated with parallel early increase (not shown) and late decrease (Fig. 1A) in cell fractional shortening. To determine the respective role of TNFRI and TNFRII pathways in TNFα effects, neutralizing Mabs, specific for TNFRI or TNFRII, were added to cardiac myocytes, 30 min before subsequent addition of TNFα. As shown in Fig. 1B, TNFR1- and TNFR2-Mabs exerted dose-dependent opposite effects on TNFα-induced ROS production, with a full inhibitory effect triggered in the presence of 3 μg/ml anti-TNFRI-Mab, contrasting with a maximal 286 ± 73% amplification elicited by 3 μg/ml anti-TNFRII-Mab. Neutralizing anti-TNFRI Mab, which inhibited ROS production also abrogated the dual early positive and late negative effect of
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TNFα on the amplitude of 

$[Ca^{2+}]_{i}$ transients (Fig. 1C), uncovering a new persistent TNFα-induced positive increase in the amplitude of 

$[Ca^{2+}]_{i}$ transients, with a mean 55 ± 7% stimulation over basal after 30 min, associated with a parallel increase in cell fractional shortening (not shown). Likewise, in cardiac myocytes isolated from rats treated for 2 weeks with the antioxidant molecule, NAC, TNFα failed to induce ROS generation, producing 7 ± 2% of the maximal H2O2 response as compared with 35 ± 6% in cardiac myocytes isolated from control rats. In the absence of ROS production, TNFα elicited a persistent increase in the amplitude of 

$[Ca^{2+}]_{i}$ transients, with a mean 58 ± 8% stimulation over basal after 30 min (Fig. 1C). In contrast, cardiac myocytes in vitro treated with neutralizing TNFR1-Mab suppressed the deleterious effect of TNFα and 11 ± 4 and 7 ± 2% nonrod-shaped cells were counted in the absence and in the presence of TNFα, respectively (Fig. 1D). In
vivo treatment of rats with NAC before cell isolation also protected cardiac myocytes from TNFα-induced cell death since 18 ± 8 and 24 ± 4% nonrod-shaped cells were counted in the absence and in the presence of TNFα, respectively (Fig. 1D). In contrast, treatment with TNFR2-Mab exacerbated the deleterious effect of TNFα on cell survival with 17 ± 4 and 68 ± 6% nonrod-shaped cells counted in the absence and in the presence of TNFα, respectively. Taken together, these results supported the important role of the TNFR1/ROS pathway in cardiac myocytes but also highlighted a major protective effect of TNFR2 signaling. Interestingly, in regard to TNFα responses, the in vivo NAC treatment reproduced TNFR1-Mabs action, neutralizing TNFR1 and unmasking TNFR2. Note that, compared with control rats, NAC-treated rats displayed similar undetectable cardiac TNFα levels (<5 pg/mg prot) and comparable cardiac TNFR1 (6.3 ± 0.47 and 5.1 ± 0.4 pg/mg prot, respectively) and TNFR2 protein expression (12.4 ± 1.22 and 8.7 ± 0.5 pg/mg prot, respectively), giving a similar TNFR1/TNFR2 ratio (0.5 ± 0.01 and 0.58 ± 0.03, respectively). To characterize TNFR2-dependent mechanisms underlying the persistent positive effect of TNFα on [Ca2+] handling and cell fractional shortening, we used cardiac myocytes isolated from NAC-treated rats as a trick to silence TNFR1 pathways.

In Cardiac Myocytes Isolated from NAC-treated Rats, TNFα Enhanced [Ca2+] Transients and Cell Fractional Shortening via TNFR2-dependent Activation of ERK, MSK1, cPLA2, PKCζ, CaMKII, and Resultant Selective Phosphorylation of the Thr17-PLB Residue—As shown in Fig. 2, in cardiac myocytes isolated from NAC-treated rats, TNFα increased the amplitude of [Ca2+] transients and cell fractional shortening via TNFR2, cPLA2, and PKCζ activation. Cardiac myocytes isolated from NAC-treated rats (NAC CM) were pretreated for 30 min without or with anti-TNFR1- or anti-TNFR2-Mab antibodies. After washing, cardiac myocytes were loaded with Fura2-AM alone, incubated for 10 min without or with MAFP or PS-PKζζ, electrically stimulated at 0.5 Hz, and exposed for 30 min to TNFα, as described under “Experimental Procedures.” Typical traces of [Ca2+] transients and cell fractional shortening were recorded continuously. Amplitude of [Ca2+] transients was normalized to control values determined at time 0. Mean ± S.E. of effects observed on at least 10 cells obtained from three different isolations (A) (*, p < 0.05 TNFα versus basal; #, p < 0.05 treatment versus no treatment or representative tracings (B) are presented.)
from NAC-treated rats, amplification of [Ca^{2+}]_i transients and cell fractional shortening, in response to a 30-min perfusion with TNFα, was unaffected by a pretreatment with anti-TNFR1-Mabs but blunted in the presence of neutralizing antibodies selectively directed against TNFR2 (Fig. 2, A and B). Among candidates likely to transduce positive TNFα responses, we focused on nitric-oxide synthase (NO synthase) and cytosolic phospholipase A₂ (cPLA₂) activities. In fact, both NO and AA have been shown to elicit positive contractile responses, at low doses, in cardiac myocytes (19, 21, 22). TNFα effects were unaffected by the presence of 1 mM L-NAME, the NO synthase inhibitor (not shown). In contrast, preincubation with the cPLA₂ inhibitor, MAFP, suppressed TNFα-induced responses (Fig. 2, A and B). In the same line, inhibition of PKCζ, a recently identified target of cPLA₂ in cardiac cells (23), also blunted TNFα-induced positive effects (Fig. 2, A and B).

Western blot analyses were performed to identify upstream and downstream signaling events in the cPLA₂ activation in response to TNFα. Particular attention was payed to ERK and MSK1, previously identified as key elements of cPLA₂ activation in response to ATP and β₂-adrenergic stimulation, in cardiac cells (18, 24). TNFα induced a time-dependent phosphorylation of both ERK (Fig. 3A) and MSK1 (Fig. 3B).

cPLA₂ activation in response to TNFα was illustrated by direct assessment of the AA release, measured in cardiac myocytes labeled for 24 h with [³H]AA. As shown in the right panel in Fig. 3C, basal release of AA was linear during the 20-min period examined. TNFα elicited a mean 33 ± 6% increase in the rate of AA release (Fig. 3C, right panel). Pretreatment with MAFP suppressed TNFα-induced AA release (not shown). In addition, confocal microscopy in cardiac myocytes immunostained with a cPLA₂ antibody clearly highlighted a redistri-
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bution of the cPLA2 in response to a 10-min treatment with TNFα that was visualized as an intensification of fluorescent labeling (Fig. 3C, middle panel, c compared with b). In contrast, the phosphorylation of the Ser505 residue of the cPLA2, which is currently referred to as an index of cPLA2 activation, was not observed in response to TNFα, under conditions in which the tumor promoter, TPA, was effective (Fig. 3C, left panel). The role of PKCζ as a target of TNFα action was confirmed by a time-dependent induction of its phosphorylation level in response to the cytokine (Fig. 3D).

Finally, we examined PLB phosphorylation as a possible cascade signaling terminal component directly linked to the positive effects of TNFα on [Ca2+]i, transients and cell contraction, in cardiac myocytes isolated from NAC-treated rats. As shown in Fig. 3F, TNFα induced a time-dependent phosphorylation of PLB on the Thr17 residue (PT17). Surprisingly, TNFα did not elicit phosphorylation of PLB on Ser16 residue (PS16) (not shown). Note that control experiments performed in parallel with a β-AR agonist revealed efficient and predominant phosphorylation of PS16-PLB in cardiac myocytes (not shown). We also demonstrate the critical impact on TNFα phosphorylation of PS16-PLB in cardiac myocytes isolated from NAC-treated rats (not shown). TNFα also produced phosphorylation of CaMKII, which ensures PT17-PLB phosphorylation in cardiac myocytes (Fig. 3E) (25).

As shown in Fig. 4A, the cascade of TNFα-induced ERK, MSK1, PKCζ, CaMKII, and PLB phosphorylations was blunted upon TNF2 redistribution but insensitive to the presence of anti-TNF2 Mab antibodies. Similarly, the TNFα-induced redistribution of the cPLA2, and the increase in [3H]AA release appeared to be TNF2-dependent but TNF1-independent events (Fig. 4B).

PKCζ, CaMKII, and PLB phosphorylations, triggered by TNFα, were sensitive to MAFP treatment, indicating that they occurred downstream of cPLA2 activation (Figs. 5 and 6). In contrast, MAFP treatment did not suppress the effect of TNFα on either ERK or MSK1 phosphorylation and a 166 ± 5% and a 168 ± 38% increase in the level of ERK and MSK1 phosphorylation were induced in response to the cytokine, respectively. Noteworthy, the effect of TNFα on the phosphorylation of CaMKII was not affected by the inhibitor of PKCζ, with a 147 ± 9% increase in the level of CaMKII phosphorylation in response to TNFα to be compared with 151 ± 9% in the absence of inhibitor, clearly arguing for CaMKII and PKCζ activations as two independent signaling events.

DISCUSSION

This study defines the role of TNF1 and TNF2 with respect to TNFα effects in cardiac myocytes on ROS production, Ca2+ signaling, cell fractional shortening, and cell survival. We also demonstrate the critical impact on TNFα signaling in cardiac myocytes of an in vivo treatment with the glutathione precursor NAC. NAC treatment is proving a valuable tool to promote and provide new insights into the mechanisms that contribute to TNF2 signaling.

Our results indicate that NAC treatment blunts TNF1-dependent production of ROS, dual positive and negative action on both [Ca2+]i, handling and cell fractional shortening, and alteration of cell survival. A consequence of TNF1 neutralization by in vivo NAC treatment is the emergence of TNF2 signaling and unmasking of a strong stimulatory effect of TNFα on both [Ca2+]i, handling and cardiac myocyte fractional shortening. Analysis of the TNF2 signaling in cardiac myocytes isolated from NAC-treated rats identifies ERK, MSK1, cPLA2, PKCζ, CaMKII, and PLB as the cascade signaling of TNFα (Fig. 6). In addition, we highlight a
selective Thr\textsuperscript{17} phosphorylation of PLB following TNF\textsubscript{R2} stimulation.

NAC appears as a unique tool to elucidate cardiac TNF\textsubscript{R2} signaling. In fact, TNF\textsubscript{R}\textalpha-induced phosphorylations of ERK, MSK1, PKC\textgreek{z}, CaMKII, and PLB were almost undetectable in cardiac myocytes isolated from control rats, probably due to the concomitant dominant opposite impact of the TNF\textsubscript{R1} pathway on these targets. Direct indications of TNF\textsubscript{R}\textalpha-induced TNF\textsubscript{R2} activation in cardiac myocytes isolated from control rats were restricted to TNF\textsubscript{R2}-dependent \textsuperscript{3}HAA release similar to that produced in cardiac myocytes isolated from NAC-treated rats (not shown). Other evidences remained indirect but argued for a critical regulation of TNF\textsubscript{R1}-activated pathways by TNF\textsubscript{R2}. Thus: (i) the positive effect of TNF\textsubscript{R}\textalpha on [Ca\textsuperscript{2+}] handling and cell fractional shortening, unraveled after anti-TNF\textsubscript{R1} Mab treatment, was neutralized by anti-TNF\textsubscript{R2} Mabs, (ii) TNF\textsubscript{R}\textalpha-induced ROS production was amplified by anti-TNF\textsubscript{R2} Mabs, and (iii) the deleterious effect of TNF\textsubscript{R}\textalpha on cell survival was exacerbated by anti-TNF\textsubscript{R2} Mabs.

Taken together, our results clearly point out the stimulation of the TNF\textsubscript{R2} receptor subtype in response to TNF\textsubscript{R}\textalpha. However, all experiments were performed using human sTNF\textsubscript{R}\textalpha, which has been considered as efficiently active on TNF\textsubscript{R1} only, in contrast to membrane-bound TNF\textsubscript{R}\textalpha that is able to activate both TNF\textsubscript{R1} and TNF\textsubscript{R2}. Long term NAC treatment could favor human sTNF\textsubscript{R}\textalpha binding to TNF\textsubscript{R2}. However, TNF\textsubscript{R1}-independent and TNF\textsubscript{R2}-dependent activation of \textsuperscript{3}HAA release, which was measured in response to human sTNF\textsubscript{R}\textalpha, was similar in cardiac myocytes isolated from either control (data not shown) or NAC-treated rats. In fact, recent flow cytometry based assays gave evidence for the interaction of human sTNF\textsubscript{R}\textalpha with mouse TNF\textsubscript{R2} (26).

Essentially considered as a cardiodepressant mediator, TNF\textsubscript{R}\textalpha in vivo elicits a delayed and marked negative inotropic effect on cardiac contraction, that is however preceded by an early and limited positive inotropic effect (27, 28). TNF\textsubscript{R}\textalpha-negative cardiac effects would result from disturbance of Ca\textsuperscript{2+} homeostasis, disruption of excitation-contraction coupling, desensitization of the \beta-receptor as well as feedback-induction of other myocardial depressants such as IL1-\beta. Our results show a clear association of negative effects of TNF with ROS production (28).

According to the literature, the TNF\textsubscript{R1} receptor subtype clearly mediates major signaling mechanisms by which TNF\textsubscript{R}\textalpha influences cardiac function in normal heart, overwhelming functional expression of the TNF\textsubscript{R2} receptor subtype. However, studies using mice lacking either TNF\textsubscript{R1} or TNF\textsubscript{R2} or both receptors, suggest that, not only TNF\textsubscript{R1}, but also TNF\textsubscript{R2}, participate in the pathophysiology of heart failure (12). Studies using double transgenic mice with cardiac-specific overexpression of TNF\textsubscript{R}\textalpha and TNF\textsubscript{R1} or TNF\textsubscript{R2} deletion have demonstrated that alterations in the balance of TNF\textsubscript{R1} and TNF\textsubscript{R2} signal transduction pathways, defined the severity of TNF\textsubscript{R}—
induced heart failure and cardiac remodeling. TNFR1 activation would promote adverse remodeling whereas TNFR2 would mediate cardioprotective effects (12). Our data clearly argue for a predominant role of TNFR2 in mediating TNFα effects in cardiac myocytes obtained from NAC-treated rats, or from control rats after neutralization of TNFR1.

In contrast with the signaling of TNFR1-mediated negative effects of TNFα, knowledge on signaling mechanisms mediating the positive effects of the cytokine remains sparse. Our results argue for the role of a TNFR1-induced ROS production associated with the early transient positive response elicited by TNFα in cardiac myocytes isolated from control rats. Note that ROS production has already been associated with positive inotropic effect of endothelin in cardiac myocytes, via Na⁺/H⁺ exchanger stimulation and Na⁺/Ca²⁺ exchanger reverse mode activation (29). In contrast, the delayed positive effect of TNFα released after TNFR1 neutralization or NAC treatment clearly relies on ROS-independent activation of TNFR2. We have previously reported that TNFα-induced short-term activation of cPLA₂ supported a positive effect of the cytokine in cardiac myocytes isolated from control rats (19). In the present study we provide evidence that TNFα activates cPLA₂ via TNFR2. Note that phosphorylation of the cPLA₂ on Ser⁵⁰⁵ has long been considered as a prerequisite for cPLA₂ activation. Accordingly, TNFR2 used to be claimed unrelated to activation of the enzyme. Only recently, a study performed by the group of MacEwan highlighted distinct regulations of cPLA₂ phosphorylation, translocation, proteolysis, and activation by TNFR subtypes. cPLA₂ stimulation by TNFR2 was shown to be unrelated to Ser⁵⁰⁵ phosphorylation of the enzyme, in contrast to the TNFR1-dependent regulation (30). The absence of TNFR2-induced cPLA₂ Ser⁵⁰⁵ phosphorylation and our finding that MSK1 mediates TNFR2-induced cPLA₂ stimulation, agree with previous observation that β₂-adrenergic- and ATP-induced cPLA₂ activation, both mediated by MSK1, occurred in the absence of Ser⁵⁰⁵ phosphorylation, (18).

We previously located cPLA₂ in caveolae/sarcoplasmic reticulum functional platforms together with MSK1, PLB, and SERCA that are major effectors of Ca²⁺ cycling (18). Our results clearly show that TNFR2 increases the amplitude of [Ca²⁺], transients and cell fractional shortening in cardiac myocytes. PLB phosphorylation on Ser¹⁶ or Thr¹⁷ residues leads to the release of SERCA inhibition exerted by unphosphorylated PLB and determines the contractile function in cardiac myocytes. Ser¹⁶ PLB phosphorylation by PKA, that triggers an increase in [Ca²⁺], and subsequent activation of CaMKII, potentiates Thr¹⁷ PLB phosphorylation by CaMKII, in particular in response to β-adrenergic stimulation. However, Ser¹⁶ and Thr¹⁷ PLB can be phosphorylated independently, and both phosphorylations contribute to the contractile function in cardiac myocytes (31). Thr¹⁷ PLB phosphorylation state is the result of phosphorylation by CaMKII and dephosphorylation by protein phosphatase 1 (PP1) (25). We identify CaMKII and Thr¹⁷ PLB phosphorylations downstream of TNFR2-induced cPLA₂ activation. Thr¹⁷ PLB phosphorylation occurs independently of Ser¹⁶ PLB phosphorylation. Of note, recent evidence indicates that Thr¹⁷ PLB phosphorylation on its own participates in a protective mechanism that favors Ca²⁺ handling and limits intracellular Ca²⁺ overload and is implicated in the mechanical recovery under some pathological conditions, like acidosis and stunning (25).

We show that PKCζ activation also participates in the selective phosphorylation of Thr¹⁷ PLB in response to TNFα, independently of CaMKII action, because treatment with the PKCζ inhibitor suppresses TNFα-induced Thr¹⁷ PLB phosphorylation (186 ± 26% versus 105 ± 4% of the control level, in the absence and in the presence PS-PKCζ, respectively) without affecting TNFα-induced CaMKII phosphorylation. PKCζ are Ser/Thr protein kinases, members of the atypical group of PKCs, characterized by insensitivity to both diacylglycerol and calcium, but activation by other phospholipid cofactors such as AA or ceramide (32). One hypothesis might be that PKCζ activation, downstream cPLA₂ activation, favored Thr¹⁷ PLB phosphorylation via protein phosphatase inhibition. In fact, in smooth muscle cells, AA-induced activation of PKCζ triggers PP1 inhibition (33).

Force et al. (23) reported a hypertrophy of cardiac and skeletal muscles in a mouse model genetically invalidated for cPLA₂. The authors concluded to the negative regulation of IGF₁ signaling by cPLA₂, and the implication of PKCζ as a crucial target for cPLA₂ (23). PKCζ has also been shown to play a pivotal role in the catabolic pathways initiated by proinflammatory cytokines, IL-1β and TNFα (34). Our study points out a beneficial role of PKCζ in cardiac myocytes, observed in the absence of ROS production and supporting positive impact on Ca²⁺ handling and cell fractional shortening. Its additional potential protective role against alteration of cell survival warrants further examination since a recent study provided evidence that PKCζ abrogated proapoptotic action of Bax via phosphorylation (35). In contrast, a deleterious role of PKCζ on cardiac function was reported in ischemia-reperfusion injury which is, in particular, characterized by ROS-induced oxidative stress (36). Hence, defining PKCζ cardiovascular impact is of major importance, all the more as enzyme inhibition has been proposed as a therapeutic option for the chronic treatment of osteoarthritis (37).

In the present study, possible direct inhibition of TNFα binding to TNFR1 by NAC (38), or direct antioxidant effect of the NAC molecule, could be ruled out because NAC was given to the rats per os, for 2 weeks, but was absent from all experiments performed in isolated cardiac myocytes. More likely, in vivo NAC treatment resulted in an increased intracellular glutathione level, as previously described (16). Neutralization of the TNFα-induced TNFR1-dependent depressant effect might derive from glutathione-induced sphingomyelinase inhibition and glutathione antioxidant action. Thus, our results argue in favor of glutathione as an anti-inflammatory compound, combining anti-TNFα and pro-TNFα properties. Because inflammation has been clearly linked to cardiovascular disorders (8), the use of NAC as an anti-inflammatory drug precursor, and not as a mere antioxidant, warrants evaluation. In addition, our study contributes to the elucidation of TNFR2-mediated pathways in the cardiac myocyte, and may provide novel insights into the role of TNFα and/or TNFRα receptors as targets for therapeutic interventions in patients with heart failure.
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