Sphingosine Mediates the Immediate Negative Inotropic Effects of Tumor Necrosis Factor-α in the Adult Mammalian Cardiac Myocyte*

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To determine whether activation of the neutral sphingomyelinase pathway was responsible for the immediate (30 min) negative inotropic effects of tumor necrosis factor-α (TNF-α), we examined sphingosine levels in diluent and TNF-α-stimulated cardiac myocytes. TNF-α stimulation of adult feline cardiac myocytes provoked a rapid (15 min) increase in the hydrolysis of [14C]sphingomyelin in cell-free extracts, as well as an increase in ceramide mass, consistent with cytokine-induced activation of the neutral sphingomyelinase pathway. High performance liquid chromatographic analysis of lipid extracts from TNF-α-stimulated cardiac myocytes showed that TNF-α stimulation produced a rapid (30 min) increase in free sphingosine levels. Moreover, exogenous D-sphingosine mimicked the effects of TNF-α on intracellular calcium homeostasis, as well as the negative inotropic effects of TNF-α in isolated contracting myocytes; time course studies showed that exogenous D-sphingosine produced abnormalities in cell shortening that were maximal at 5 min. Finally, blocking sphingosine production using an inhibitor of ceramidase, n-oleoylthanolamine, completely abrogated the negative inotropic effects of TNF-α in isolated contracting cardiac myocytes. Additional studies employing biologically active ceramide analogs and sphingosine 1-phosphate suggested that neither the immediate precursor of sphingosine nor the immediate metabolite of sphingosine, respectively, were likely to be responsible for the immediate negative inotropic effects of TNF-α. Thus, these studies suggest that sphingosine mediates the immediate negative inotropic effects of TNF-α in isolated cardiac myocytes.

Tumor necrosis factor-alpha (TNF-α) is a proinflammatory cytokine that has been implicated as a potential pathogenetic mechanism for cardiac disease states wherein left ventricular dysfunction supervenes, including systemic sepsis (1), acute viral myocarditis (2), cardiac allograft rejection (3), myocardial reperfusion injury (4), and congestive heart failure (5). The long-standing interest in defining the mechanisms responsible for the cardiodepressant effects of TNF-α has been intensified recently by experimental studies that have shown that TNF-α produces negative inotropic effects in the intact left ventricle (6, 7), in thin strips of myocardial tissue (8), and in isolated contracting cardiac myocytes (6). Although the exact cellular signaling pathways that are responsible for the negative inotropic effects of TNF-α are not known, a careful inspection of the literature suggests that TNF-α modulates myocardial function through at least two different pathways.

It is quite clear, for example, that TNF-α can produce immediate negative inotropic effects in myocardial tissue within 10–30 min (6, 8). Similarly, it is equally clear that TNF-α exerts delayed effects on myocardial function that appear to be related to uncoupling of the β-adrenergic receptor from cyclic AMP, rather than from a direct depression in basal myocardial contractility per se; moreover, these effects occur only after prolonged TNF-α exposure (24–72 h) (9, 10). Given the recognition that TNF-α increases nitric oxide (NO) levels in myocardial tissue through increased transcription of the inducible Ca2+-independent form of nitric oxide synthase (NOS) (11, 12), given that NO directly mediates myocardial depression (13, 14), and given that NO is likely responsible for the uncoupling of the β-adrenergic receptor following TNF-α stimulation (15), the logical assumption has been that NO mediates the full spectrum of cytokine-induced cardiodepressant effects. However, no previous report to date has provided direct evidence that shows that TNF-α stimulates NO production in cardiac myocytes with a time course that is rapid enough to explain the immediate negative inotropic effects of TNF-α (6). As a case in point, a recent study in which thin strips of myocardial tissue from Syrian hamsters were treated with TNF-α provided indirect evidence that suggested that the immediate (<5 min) negative inotropic effects “appear(ed) to result from enhanced activity of a constitutive (Ca2+-dependent) NO synthase enzyme in the myocardium.” (8) Nonetheless, although combinations of cytokines may increase Ca2+-dependent NOS activity indirectly over 24 h by increasing the synthesis of NOS cofactors (16), the demonstration of a rapid increase in Ca2+-dependent NOS activity by TNF-α, or by any other cytokine, has not been observed thus far (17). Moreover, we have found that the immediate negative inotropic effects of TNF-α were not abrogated by NOS inhibition (6), suggesting that TNF-α may produce myocardial depression through a NOS-independent pathway.

During the course of previous studies we observed that TNF-α-induced activation of the type 1 TNF receptor (TNFR1) resulted in reversible negative inotropic effects in isolated cardiac myocytes as a direct result of alterations in intracellular calcium homeostasis (6, 18). Insofar as concentrations of TNF-α that produced negative inotropic effects did not produce dis-
cerneline changes in the voltage-sensitive inward calcium current, we suggested that the TNF-α-induced alterations in intracellular calcium homeostasis were secondary to alterations in sarcoplasmic reticular handling of calcium (6). Encouraged by the observation that TNF-α-induced oligomerization of TNFR1 leads to the rapid degradation of sphinomyelin (19) with the resultant generation of a sphingoid base termed sphingosine (20), as well as by the observation that sphingosine was not only present in cardiac and skeletal muscle (21), but was also capable of blocking calcium release from the ryanodine receptor (22, 23), we investigated whether the immediate negative inotropic effects of TNF-α were mediated by sphingosine. In the present brief report we demonstrate that sphingosine is both necessary and sufficient to produce the negative inotropic effects of TNF-α in isolated cardiac myocytes, thus suggesting that TNF-α-induced activation of the neutral sphingomyelinase pathway is responsible for the immediate negative inotropic effects of this proinflammatory cytokine.

**EXPERIMENTAL PROCEDURES**

*Cell-free Sphingomyelinase Assay—*Acidic (pH 5.0) and neutral (pH 7.5) sphingomyelinase activity were measured in adult feline cardiac myocytes according to the method described by Machleidt et al. (24). Briefly, feline cardiac myocytes were isolated, and a 2-ml suspension of cells was plated at a final concentration of 5 × 10⁴ cells/ml onto laminin-coated (20 μg/ml) polystyrene Petri dishes as described previously (25, 26). On the 1st day in culture the M199 medium was changed, and the cells were treated for 0, 5, 15, 30, and 60 min with diluent (endotoxin-free 0.1% human serum albumin) or with recombinant human TNF-α (200 units/ml). The cells were then lysed, and the resultant cell supernatants were incubated at 37 °C with 1 μl of [methylv-¹⁴C]sphinomyelin (25 μCi/ml). The reaction was stopped after 2 h by the addition of chloroform:methanol (2:1). After thorough mixing by inversion and vortexing, ddH₂O was added, and the two phases were separated by centrifugation. The upper aqueous phase containing [¹⁴C]phosphorylcholine was removed and counted in a liquid scintillation counter. Final results were expressed as fold increase in [¹⁴C]phosphorylcholine levels relative to base-line levels.

*Ceramide Mass (Diacylglycerol Kinase Assay)—*Ceramide mass was measured according to the method of Preiss et al. (27). Briefly, isolated cardiac myocytes were prepared as described above and stimulated for 30 min with TNF-α (200 units/ml), either in the presence or absence of a specific inhibitor of ceramidase: n-oleylthanolamine (NOE) (28). The lipid fractions from cell extracts were extracted with chloroform/methanol (1:1 (v/v)), the samples vortexed, and then separated by centrifugation. The upper aqueous phase was aspirated and used for determination of the protein concentration, whereas the chloroform phase was dried in vacuo. The extracts were resuspended and incubated with β-hexadecylceramide diacylglycerol kinase (β-HC-DGK) (100 units) and commercial diacylglycerol kinase assay system (Amersham Corp.). Ceramide 1- phosphate was then isolated by thin layer chromatography (TLC) using chloroform/methanol/glacial acetic acid (65:15:5 (v/v/v)) in the presence of a ceramide standard, which was run simultaneously along with the unknown samples. Authentic ceramide 1-phosphate was identified by autoradiography, and the spots corresponding to ceramide 1-phosphate were scraped from the plates and then counted in a scintillation counter. Final results were expressed as cpm/mg protein.

*Sphingosine Analysis by Liquid Chromatography—*Sphingosine Analysis by Liquid Chromatography—O-Phthalaldialdehyde derivatives of the unknown samples were separated by reverse phase HPLC using an isocratic elution with methanol, 5 mM potassium phosphate (pH 7.0) (90:10), employing a Beckman 112 solvent delivery module, a 250 × 4.6 mm C18 (5 μm) Brownlee column with a Dynamax C18 guard column; all samples and standards were run at a solvent flow rate of 1 ml/min. Fluorescent derivatives were detected using Waters 420-AC fluorescence detector with excitation and emission wavelengths of 340 and 455 nm, respectively. Under the above conditions, incorporated fluorescence is linearly related to sphingoid base levels between 2 to 400 pmol. To determine the absolute level of sphingosine in unknown samples, the amount of fluorescence was compared with a standard curve generated from known quantities of sphingosine. To confirm the level of sphingosine in diluent and TNF-α-stimulated samples, we expressed the amount of free sphingosine present in the unknown samples as the ratio of the area under the sphingosine peak to the area under the tetradecylamine peak.

**Effect of TNF-α on Free Sphingosine Levels—**To determine whether stimulation with TNF-α would increase the level of free sphingosine in isolated adult cardiac myocytes, we first determined that concentrations of NOE (10.0 μM) completely inhibited cell motion; therefore, 1.0 μM NOE was chosen as the maximal concentration of NOE to inhibit ceramidase. As an additional control for the above experiments NOE-pre-treated cells (1.0 μM) were stimulated with 200 units/ml of TNF-α and 1 μM D-sphingosine for 30 min. Second, to determine whether exogenous D-sphingosine would mimic the negative inotropic effects of TNF-α, freshly isolated cardiac myocytes were allowed to stabilize for 1 h and were then treated for 30 min at 37 °C with 200 units/ml of TNF-α, either in the presence or absence of a specific inhibitor of ceramidase, n-oleylthanolamine (NOE) (29). In preliminary control experiments we determined that concentrations of NOE >10.0 μM completely inhibited cell motion; therefore, 1.0 μM NOE was chosen as the maximal concentration of NOE to inhibit ceramidase. Isolated adult cardiac myocytes were determined according to the method of Merrill et al. (29). Briefly, lipid extracts were obtained from freshly isolated cardiac myocytes by thorough mixing the cells with chloroform/methanol (1:2) for 5 min. In order to account for variability in the lipid extraction process, 0.6 nmol of tetradeclane was added to the unknown samples as an internal standard (30). Equal volumes of chloroform and 1 M NaCl were then added, and the two phases were separated by centrifugation. The upper aqueous phase was discarded, and the chloroform phase was washed twice with 1 M NaCl and vacuum-dried for 30 min. The dried lipid extracts were then subjected to a modified alkaline hydrolysis procedure in 0.1 M KOH at pH 11, 1 M NaCl, and then vacuum-dried. Thereafter, samples were derivatized with o-phthalaldehyde, exactly as described by Merrill and colleagues (29).
inhibited PC-PLC activity completely, we also pretreated the cells with 20 μM sangivamycin, which has been shown to significantly inhibit diacylglycerol-dependent protein kinase C (PKC) activity (35); 20 μM sangivamycin did not significantly inhibit cell motion in preliminary control experiments. For the studies with TNF-α and sangivamycin, the cells were pretreated with inhibitor for 60 min and were then stimulated with TNF-α or diluent for an additional 30 min prior to assessing cell motion. In addition to these indirect studies, we performed direct measurements of PKC activity in TNF-α and sphingomyelin-stimulated cardiac myocytes. Evidence for cytokine-induced activation of PKC (translocation) was determined in freshly isolated myocytes stimulated for 1, 7.5, or 30 min either with 200 units ml⁻¹ TNF-α using differential membrane centrifugation and immunoblot analysis, as described previously (36); cells stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) served as the appropriate positive controls. Two separate primary antibodies were employed for immunoblotting; the first antibody was specific for PKCε, the major PKC isoform in myocytes, whereas the second antibody recognized PKCζ, β, γ, and δ. The relative amounts of protein kinase C in the membrane and cytosolic fractions were assessed by laser densitometry and expressed as a ratio of membrane-to-cytosolic PKC. Evidence for inhibition of PKC activity was sought by determining the incorporation of [³²P]ATP into a PKC-specific pseudosubstrate peptide, as described previously (37). Briefly, cardiac myocytes were homogenized and then centrifuged for 1 h at 1,000 × g; the resultant crude homogenate was then separated into cytosolic and membrane fractions by centrifugation at 70,000 × g. Insofar as preliminary studies showed that ~75% of the PKC activity resided in the cytosolic fraction, this fraction was employed for all further studies. The PKC inhibitory activity of TNF-α (200 units ml⁻¹) and sphingosine (0.1–10 μM) was compared with that of 100 nM staurosporine in assays wherein myocyte cytosolic extracts were stimulated with mixed micelles containing 0.3 mg/ml phosphatidylserine and 24 μg/ml PMA, in order to simulate PKC activity.

**Intracellular Calcium Homeostasis**—Intracellular calcium transients were determined in isolated contracting cardiac myocytes using the fluorescent indicator fluo-3 AM (20 μM), exactly as we have described previously (6). A time-intensity curve for fluorescence brightness was determined for a single cardiac myocyte contraction by measuring the total fluorescence brightness over the surface area of individual cell; the final time-intensity curves were determined as the average values for 10 consecutive contraction sequences after cell shortening had stabilized. For the purpose of comparison between v-sphingosine (1 μM) and diluent-treated cells, peak values of intracellular fluorescence brightness were compared.

**Statistical Analysis**—Data are expressed as mean ± S.E. Data were analyzed by one-way analysis of variance, with post hoc testing where appropriate (Dunnett’s), or by non-paired t tests. **RESULTS AND DISCUSSION**

**TNF-α-induced oligomerization of TNFR1** has been shown to activate membrane-bound neutral sphingomyelinase, with the resultant generation of ceramide, which can then be deacylated to sphingosine by the enzyme ceramidase (28). Three series of experiments were performed to determine whether TNF-α activated this pathway in the adult mammalian cardiac myocyte. Table I shows that TNF-α stimulation resulted in time-dependent increase in neutral sphingomyelinase activation in adult cardiac myocytes. As shown, [¹⁴C]phosphorylcholine levels were significantly different from control values by 15 min (p < 0.05) and were ~2-fold greater than control values 60 min following stimulation. TNF-α stimulation also activated the acidic sphingomyelinase pathway, consistent with reports from other laboratories (34). Next we measured ceramide mass in diluent and TNF-α-stimulated cardiac myocytes (n = 4 dishes per group). When compared with diluent-treated controls, TNF-α stimulation provoked a significant increase in ceramide mass (10.458 ± 571 versus 18.955 ± 831 pmol/10⁶ cells). In contrast, stimulating the cells with diluent in the presence of NOE resulted in ~8-fold increase in ceramide mass (82.998 ± 5.140 cpm/μg protein), whereas stimulating the cells with TNF-α in the presence of NOE resulted in ~12-fold increase in ceramide mass (121.155 ± 24.086 cpm/μg protein). Thus, these studies are consistent with the notion that NOE acts, at least in part, by inhibiting ceramidase (28). Finally, to determine whether TNF-α activation would stimulate increased levels of free sphingosine in cardiac myocytes, freshly isolated cells were stimulated with TNF-α for 30 min before lipid extraction. Fig. 1 shows the elution profiles for the α-phthalaldehyde derivatives of the lipid extracts from cardiac myocytes stimulated with diluent (Fig. 1A) and 200 units ml⁻¹ of TNF-α (Fig. 1B). As shown, a major sphingosine peak was resolved at 14 min in the diluent and TNF-α-treated myocytes, consistent with previous studies that have demonstrated constitutive levels of sphingosine in cardiac and skeletal muscle (21). The identity of the sphingosine peak was confirmed by its comigration on HPLC with authentic sphingosine standards, as well as by the observation that closely related exogenous lipids (dihydrosphingosine and psychosine) had different elution times when added to the unknown samples. In preliminary control experiments we established that the amount of free sphingosine present in isolated cardiac myocytes was ~23 pmol/10⁶ cells, consistent with reports in cardiac and skeletal muscle, as well as other cell types (29, 38, 39). As shown in Fig. 1 the elution profile for tetradeaclyamine, which was added as an internal standard, had a retention time of 30 min. In the representative example depicted in Fig. 1B, TNF-α stimulation led to a 1.4-fold increase in the sphingosine/TDA ratio when compared with values obtained in diluent-treated cardiac myocytes.

**TABLE I**

|                   | Neutral sphingomyelinase | Acidic sphingomyelinase |
|-------------------|--------------------------|-------------------------|
| 0 min             | 1.00 ± 0.03              | 1.00 ± 0.04             |
| 5 min             | 1.07 ± 0.03              | 1.11 ± 0.05             |
| 15 min            | 1.27 ± 0.03              | 1.27 ± 0.03             |
| 30 min            | 2.03 ± 0.03              | 1.22 ± 0.04             |
| 60 min            | 2.17 ± 0.02              | 1.11 ± 0.05             |

*p < 0.05 compared with control values (Dunnett’s test).**

Data are expressed as the fold change from base-line values (0 min). Freshly isolated cardiac myocytes were stimulated with 200 units ml⁻¹ TNF-α for the times indicated above (n = 5 dishes/time point), and the extent of sphingomyelin hydrolysis was examined under neutral (pH 7.5) and acidic (pH 5.0) conditions (see “Experimental Procedures”). One-way analysis of variance indicated that there were significant overall differences in the extent of activation of the neutral (p < 0.004) and acidic sphingomyelinas (p < 0.003) pathways. The base-line values (mean ± S.E.) for [¹⁴C]phosphorylcholine levels under neutral conditions were 1145.6 ± 29.8 cpm/mg protein and 77628.0 cpm/mg protein under acidic conditions.
sphingosine levels, similar to that which was observed with the wild type TNF-α. As shown, the TNFM2 ligand, which does not affect cell motion (18), did not increase levels of free sphingosine in isolated contracting cardiac myocytes. The findings obtained with the TNFM1 and TNFM2 ligands are consistent with previous reports that have shown that TNFR1, as opposed to TNFR2, produces negative inotropic effects in isolated contracting cardiac myocytes (18). Next, to determine whether blocking the conversion of ceramide to sphingosine would abrogate the negative inotropic effects of TNF-α, the cells were pretreated (60 min) with a specific inhibitor of ceramidase: n-oleoylthanolamine (NOE) (28). The important finding shown by Fig. 3 is that the negative inotropic effects of TNF-α were abrogated completely by pretreatment with 1.0 μM NOE. To confirm that NOE blocked the generation of free sphingosine, we measured free sphingosine levels in TNF-α-stimulated cells in the presence and absence of NOE. HPLC analysis showed that NOE pretreatment blunted the TNF-α-induced increase in free sphingosine levels by ~75%. Moreover, when the NOE-pretreated cells were stimulated concurrently with sphingosine and TNF-α, we observed a significant depression in cell shortening, suggesting that NOE did not act by interfering with the negative inotropic effects of sphingosine in isolating contracting cardiac myocytes. The salient finding shown by Fig. 4A is that treating the cells with exogenous d-sphingosine for 30 min resulted in a concentration-dependent decrease in myocyte shortening that was significantly different from control for ≥0.001 μM sphingosine. Importantly, the concentrations of exogenous d-sphingosine that were necessary to depress cell shortening fell within the theoretically calculated range for sphingosine levels in TNF-α-stimulated cardiac myocytes (~0.1–1.0 μM, assuming a cell density of 1.25×10⁶ ml⁻¹) (21, 38). To confirm the specificity of the observed effects with d-sphingosine, the cells were treated with dihydrosphingosine, which differs from sphingosine structurally by the absence of a double bond in the carbon 4–5 position (41). Fig. 4A shows that dihydrosphingosine had no effect on isolated cell shortening, thus arguing against a nonspecific lipid membrane effect of sphingosine. The inset of Fig. 4A shows that the sphingosine-induced (1 μM) depression in cell shortening was maximal at ~5 min, congruent with the overall rapid time course for the development of the immediate negative inotropic effects of TNF-α (6, 8). Finally, as shown in the inset, the negative inotropic effects of n-sphingosine were shown to be completely reversible within 30 min after n-sphingosine was washed out of the cells (Fig. 4), consistent with previous observations that the immediate negative inotropic effects of TNF-α are completely reversible (6, 8). Fig. 4B shows that when the cells were pretreated with ≥1 μM sphingosine 1-phosphate, the extent of cell shortening was reduced significantly (p < 0.05) compared with control values. The inset of Fig. 4B shows that the time course for the onset of negative inotropic effects with sphingosine 1-phosphate was delayed relative to that observed with n-sphingosine and was significantly different (p < 0.05) from control only after 30 min of continuous stimulation. Thus, the time course for the onset of negative inotropic effects in sphingosine 1-phosphate-treated myocytes...
for thenegative inotropic effects of D-sphingosine. For these studies, the motion was examined. In addition, the cells were treated with 1 mM D-sphingosine; cell motion was determined 30 min after washing the cells. The myocytes were treated for 30 min with diluent (4840 μM MD-sphingosine, and cell shortening was examined using video edge detection (6). The inset of A shows the time course for the negative inotropic effects of D-sphingosine. For these studies, the cells were treated for 5, 15, or 30 min with 1 mM D-sphingosine, and cell motion was examined. In addition, the cells were treated with 1 μM D-sphingosine for 30 min and was 6-fold slower than was observed when the cells were stimulated with D-sphingosine (5 min). Second, if sphingosine 1-phosphate mediates the negative inotropic effects of sphingosine, then one would predict sphingosine 1-phosphate would be more potent than sphingosine on a molar basis. However, as shown by Fig. 4, A and B, sphingosine 1-phosphate is 1000-fold less potent than sphingosine in terms of producing negative inotropic effects in isolated cardiac myocytes. Thus, while we cannot exclude a potential contributory role for sphingosine 1-phosphate in terms of mediating the negative inotropic effects of TNF-α, the data do not support a primary role for this molecule.

We next examined the effects of exogenous D-sphingosine on intracellular calcium transients in isolated contracting cardiac myocytes to determine whether sphingosine would mimic the effects of TNF-α on intracellular calcium homeostasis (6). Fig. 5 shows representative time-intensity curves for fluorescence brightness in cardiac myocytes treated either with 1 mM D-sphingosine or with diluent. As shown, treatment with D-sphingosine produced a striking decrease in the peak levels of intracellular fluorescence brightness, consistent with previous observations that 1 mM sphingosine is sufficient to inhibit calcium release by the sarcoplasmic reticular ryanodine receptor (22, 23). Similar findings with respect to the effects of sphingosine on intracellular calcium homeostasis have also been observed in neonatal cardiac myocytes (43). The inset of Fig. 5 summarizes the results for the studies, wherein peak fluorescence brightness was examined for groups of diluent and D-sphingosine-treated cells. As shown, there was ~50% decrease (p < 0.05) in the peak intensity of fluorescence brightness for the D-sphingosine (1 μM)-treated cells compared with diluent-treated controls, again consistent with the previous findings from this laboratory that have shown that TNF-α suppressed peak intracellular fluorescence brightness by ~40% (6).

In addition to engaging the neutral sphingomyelinase pathway, TNF-α-induced oligomerization of TNFR1 activates phosphatidylinositol-specific phospholipase C (PC-PLC), with increased activity of diacylglycerol-dependent PKC, as well as the phospholipase A2 pathway, with increased formation of arachidonic acid (44). Previously, we have shown that inhibiting arachidonic acid cyclooxygenase did not abrogate the TNF-α-induced negative inotropic effects (6), suggesting that prostag-
landins were not responsible for producing the negative inotropic effects of TNF-α. To determine whether the PC-PLC pathways was important in terms of mediating the negative inotropic effects of TNF-α, we stimulated isolated contracting cardiac myocytes with TNF-α in the presence of specific inhibitors of the PC-PLC and the diacylglycerol-dependent PKC pathways. These studies showed that the negative inotropic effects of TNF-α were not abrogated by PC-PLC inhibition with D609 nor by PKC inhibition with sangivamycin: that is TNF-α-induced a 21.2 ± 2% and 18 ± 6% decrease in myocyte shortening, respectively, in cells pretreated with 0.4 μM D609 (p < 0.002 compared with control; n = 10 cells) and 20 μM sangivamycin (p < 0.009 compared with control; n = 9 cells). We also directly measured PKC activity in TNF-α and sphingosine-stimulated cells. In unstimulated myocytes the PKC membrane-to-cytoplasm ratio was ~0.4. Stimulating the cells with 100 nM PMA resulted in a rapid (~1 min) increase in the PKC membrane-to-cytoplasm ratio to 0.8, which was maintained at 7.5 min. In contrast, there was no change in the membrane-associated PKC ratios at any time point up to 30 min for the cells stimulated with 200 units ml⁻¹ TNF-α. Identical results were obtained when an antibody that recognized PKCα, β, γ, and δ was used. Insofar as sphingosine has been shown to decrease PKC activity in certain cell types (41), we also measured incorporation of 35P into a specific PKC pseudosubstrate (n = 6 experiments/group) in PMA-stimulated cytosolic extracts from cells that had been pretreated with TNF-α (200 units ml⁻¹) or sphingosine (0.1–10 μM). This study showed that there was no significant difference in radiolabeling of the pseudosubstrate in cytosolic extracts pretreated with TNF-α (64 ± 4 pmol/min/mg) or with 0.1–10 μM sphingosine (76.0 ± 5.6, 72 ± 8.0, 76 ± 6.0 pmol/min/mg, respectively), when compared with the values obtained in control cytosolic extracts (68 ± 3.2 pmol/min/mg). In contrast, there was a significant decrease in PKC activity (p < 0.05) in the cytosolic extracts that had been pretreated with 100 nM staurosporine (32.0 ± 2 pmol/min/mg). Taken together, these latter studies suggest that neither activation of PKC through the PC-PLC pathway nor inhibition of PKC activity by sphingosine play a major role in mediating the negative inotropic effects of TNF-α.

In summary, we have provided evidence that shows that TNF-α and sphingosine both control the same events in isolated contracting cardiac myocytes: that is alterations in intracellular calcium homeostasis and negative inotropism. We have also provided data that show that the time course for and degree of free sphingosine production following cytokine stimulation is sufficient to completely mimic the negative inotropic effects of TNF-α in isolated cardiac myocytes. Finally, we have shown that TNF-α-induced sphingosine production is necessary for the negative inotropic effects of this cytokine, insofar as blocking sphingosine production through inhibition of ceramide with NOE abrogates the negative inotropic effects of TNF-α. In contrast to the findings for sphingosine, we have shown that ceramide, the immediate precursor for sphingosine, is neither necessary nor sufficient to mimic the negative inotropic effects of TNF-α and that sphingosine 1-phosphate, the phosphorylated metabolite of sphingosine, is 1000-fold less potent than sphingosine in terms of producing negative inotropic effects; moreover, the delayed onset for the biological effects of sphingosine 1-phosphate in cardiac myocytes is inconsistent with the time course for the negative inotropic effects in TNF-α. Taken together, the above results provide a rational basis for concluding that sphingosine mediates the immediate negative inotropic effects of TNF-α. Although we cannot exclude a potential contributory role for NO in terms of modulating the immediate negative inotropic effects of TNF-α, it bears reem-
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