The Role of TNFα and Sphingolipid Signaling in Cardiac Hypoxia: Evidence that Cardiomyocytes Release TNFα and Sphingosine

Amy L. Cavalli, Joseph A. Ligutti, Nicole M. Gellings, Ernesto Castro, M. Trevor Page, Robert E. Klepper, Philip T. Palade[1], Wm. T. McNutt and Roger A. Sabbadini[2]

Heart Institute and Department of Biology, San Diego State University, San Diego, (1) Department of Physiology and Biophysics, University of Texas Medical Branch at Galveston, Galveston and (2) Medlyte, Inc.

Abstract

Sphingosine (SPH) is a naturally occurring signaling molecule thought to be responsible for the negative inotropic and cardiotoxic effects of the pro-inflammatory cytokine, TNFα. When subjected to hypoxia and acidosis, Langendorff perfused adult rabbit hearts generate SPH, and isolated adult rat cardiomyocytes released TNFα and SPH into the cell-conditioned media under hypoxic conditions before hypoxia-induced cell permeabilization associated with necrosis. Cardiomyocyte SPH production in response to hypoxia was blocked by TNFRII:Fc which interferes with TNFα binding to its membrane-bound receptor, suggesting that hypoxia-triggered SPH production was TNFα dependent. Extracellular SPH was rapidly converted almost exclusively to S1P by an active sphingosine kinase present in blood components, as seen *in vitro* and in Langendorff-perfused rat hearts. These results indicate that cardiac-derived TNFα and its sphingolipid mediator, SPH, may be important extracellular signals in the heart that contribute to the pathogenesis of cardiac ischemia.

Key words: apoptosis, cardiomyocytes, carnitine, hypoxia, necrosis, sphingosine, sphingosine-1-phosphate, TNFα.

Sphingolipids are primary structural components of cell membranes that also serve as cellular signaling and regulatory molecules [1, 2]. Ceramide (CER), sphingosine (SPH) and sphingosine-1-phosphate (S1P) have been most widely studied and have recently been appreciated for their roles in the cardiovascular system [see review, [3]]. Recent work suggests that SPH may mediate the negative inotropic effects of the pro-inflammatory cytokine TNFα [4-7] through direct actions on the ryanodine receptor [8] and the L-type channel [6, 9, 10]. Moreover, SPH is a very potent inducer of apoptosis in both neonatal and adult rat cardiomyocytes [11-13].

Since the sphingomyelin signal transduction pathway is activated very early during cardiac hypoxia [14, 15], cardiac tissue-derived tumor necrosis factor alpha (TNFα) might mediate ischemia/hypoxia induced sphingolipid production. The ischemic myocardium produces TNFα [15-19], which stimulates the sphingomyelinase activity of adult cardiomyocytes [5] leading to increased intracellular SPH in cardiomyocytes [5, 6, 11, 15]. Provided with the proper stimulus (e.g., LPS, cell stretch), cardiomyocytes themselves produce substantial TNFα and secrete the cytokine into the extracellular medium [20, 21], where it may act on TNFα receptors expressed on cardiomyocytes [4, 22] in an autocrine/paracrine fashion. We have recently reported that serum levels of SPH are elevated in an experimental model of heart failure [13] and that serum SPH and S1P are elevated in patients with severe coronary artery disease [23].

The present study was undertaken to determine if TNFα and sphingolipids are produced and released by hypoxic cardiomyocytes. We report that TNFα is released from cardiomyocytes and activates the sphingomyelin signal transduction system, resulting in the release of substantial amounts of SPH into the extracellular medium as well. Platelets take up extracellular SPH and convert it to S1P, which has pleotropic effects on cardiac tissue. We propose novel roles for TNFα, SPH and S1P as extracellular signaling molecules that participate in the heart's response to ischemic stress.

Methods

Adult cardiomyocyte preparation

All procedures involving experimental animals were performed in accordance with institutional and NIH guidelines for the ethical treatment of animals. Primary cultures of adult rat cardiomyocytes were prepared es-

sentially as described previously [11], except for the use of pyrogen-free collagenase (Liberase blendzyme, Roche, Indianapolis, IN) for digestion of the rat hearts.

Adult cardiomyocytes were cultured for 3 hours in 10% FCS/DMEM until treatment at densities of 35,000 cells per plate (35mm culture dish). Prior to treatment, cells were allowed to equilibrate in warm (37^oC) Tyrode's solution for 1 hour [10]. The purity of the adult cardiomyocyte preparation was assessed as $86 \pm 1\%$ rod shaped cells after 3 hours in culture (data from 8 separate cell preparations).

Hypoxia cell culture

Cardiomyocytes were cultured on plastic dishes placed in a humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) pressurized to 0.04-0.05 psi by the particular gas mixture used in the treatment in experiments similar to those published by others [14] that demonstrated early activation of sphingomyelinase in cultured cardiomyocytes exposed to mild hypoxia. Warm (37^oC) Tyrode's solution containing 0.2mM BSA, ampicillin (50mg/mL), kanamycin (100mg/mL) and fungizone (20μg/mL) was gassed for 15 minutes with $95\%N_2/5\%CO_2$ prior to cell treatment. Control (normoxia) cells were treated the same except with $95\%O_2/5\%CO_2$ and incubated in a standard incubator. At the end of each incubation period, the cell-conditioned media was aspirated and saved for analysis.

Cell viability assays

Cardiomyocytes were stained with Hoechst bisbenzimide and propidium iodide (Sigma, St. Louis, MO) to access the viability of the cells as previously described [20]. Single cell microgel electrophoresis (the comet assay) was used to quantify the percent apoptotic cells as previously reported [11, 24].

Langendorff perfusion of rabbit hearts

New Zealand white rabbits were anesthetized for 90 seconds in a chamber containing CO_2 gas, followed by cervical dislocation. The chest cavity was opened and the ventricles heparinized with a hypodermic syringe. The heart was attached to a Langendorff apparatus via the aorta to a Teflon cannula and then perfused for 5 minutes by retrograde aortic flow (2 ml/minute) using Krebs solution [118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.54 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 5.6 mM D(+) glucose, 5 mM MOPS, 1.3 mM $CaCl_2$, pH 7.0 at 37^oC] aerated for one hour with 95% O_2/ 5% CO_2. For hypoxic/acidotic conditions, the Langendorff perfused heart was initially treated for 5 minutes with the same Krebs solution aerated with 95 % O_2/ 5% CO_2 followed by a 5 minute perfusion with the Krebs solution aerated with 100% CO_2. During hypoxia/acidotic perfusion, we determined the pH of the cardiac tissue to be 5.6, which was similar to the pH of ischemic cardiac tissue reported by others [21].

The excised rabbit ventricles were homogenized in 4 vols of 50 mM potassium phosphate buffer, pH 7.0, for 20 sec at 5^oC in a Waring blender fitted with a 30 ml stainless steel cup. Then 100 μl samples of the crude rabbit heart homogenate were placed into 2 ml tubes for analysis.

Langendorff perfusion of rat hearts

Hearts were excised from anaesthetized male Sprague-Dawley rats (350-400g) and mounted in a Langendorff perfusion system. Retrograde perfusion via the aorta was initiated at 37^oC at a constant flow of 9 mL min^{-1} with oxygenated Krebs-Henseleit buffer (25 mM $NaHCO_3$, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 1.2 mM $CaCl_2$, 11 mM glucose, BSA 1 mg mL^{-1}, pH 7.4). After each heart was suspended, a World Precision Instruments (Sarasota, FL) model # BLPR pressure transducer was introduced into the left ventricle via the Mitral valve. Each heart was stabilized for 30 minutes by perfusing with buffer. Only hearts exhibiting at least 80 mmHg left ventricular developed pressure and displaying heart rates of at least 100 bpm were subsequently treated by recirculation of 170 mL of a 20μM sphingosine solution for 90 minutes. In selected experiments, the Krebs-Henseleit buffer was supplemented with fresh (same day) human platelets obtained from the San Diego Blood Bank added to a final concentration of 7 x 10^5 platelets mL^{-1}. Control hearts were subjected to similar conditions without sphingosine or platelet addition. For all hearts, 1.2 mL aliquots of eluted samples were collected 20 minutes into the stabilization period and then every ten minutes during treatment.

High Performance Liquid Chromatography (HPLC) detection of sphingolipids

Prior to HPLC separation and quantitative analysis, sphingolipids were extracted from the cell-conditioned culture media as follows: 300 μl samples were deproteinized by adding 800 μl of warmed (70°C) butanol, vortexing and incubating at 70°C while rocking. The sample was then placed in a sonicating water bath for 10 minutes. Denatured protein and aqueous phase were separated from the butanol layer by centrifugation at 15,300 x g. The upper butanol layer was transferred and saponified by the addition of 0.5 M KOH. Samples were vortexed and then incubated at 70°C for 10 minutes, sonicated again and then centrifuged at 15,300 x g for 3 minutes. The butanol layer was decanted and dried down using a Savant (Holbrook, NY) SpeedVac and then resuspended in methanol. The extracts were then derivatized with O-phthalaldehyde (OPA) (Molecular Probes, Eugene, OR) as described by Merrill [25].

The HPLC was performed essentially as described by Merrill [25] with modifications as described previously [13, 23]. In a limited number of experiments, electron impact mass spectrometry was performed on the sphingolipids eluted by HPLC. Scattergrams were obtained by the University of California, Riverside MS Facility and were confirmed as authentic SPH and S1P by com-

paring the scatter grams we obtained to those published on the NIST national database.

Quantification of TNFα in cell-conditioned culture media

A rat TNFα-specific double-capture, high-sensitivity ELISA kit (BioSource, Camarillo, CA) was used for the determination of TNFα in the cell conditioned media.

Metabolism of sphingolipids in blood

Fresh human blood was obtained from the San Diego Blood Bank and collected in standard serum tubes containing EDTA. Care was given to prevent hemolysis of the samples. 300 μL of blood were placed in screw-capped tubes to which was added 10 μL of 2000 dpm/pmol ^{3}H-SPH (New England Nuclear, Boston MA). Samples were then vortexed for 15 seconds, and stored at 37°C in a 95% Air/5% CO_2 incubator. Incubations were terminated at the indicated time points by the addition of 800 μL of 1-butanol. The samples were briefly agitated in a bath sonicator and then placed in a 50-70°C rocking incubator for 15 minutes. Samples were then vortexed for 15 seconds, sonicated for 5 minutes, and centrifuged for 10 minutes at maximum speed in a microfuge. The butanol layer from each was then transferred and dried down using the Savant SpeedVac Plus (Holbrook, NY). The samples were then reconstituted in 50 μL of methanol and spotted on 20x20 TLC silica gel G plates (Analtec, Inc., Newark, DE). The plates were developed in butanol:acetic acid:H_2O (3:1:1) according to [23] with modifications. The plates were allowed to air dry and then stained with Ninhydrin to visualize and determine Rf values of standards spotted on the same silica plate. The plates were then monitored for tritium using a Molecular Dynamics (Sunnyvale, CA) Phosphor tritium screen and cassette and read on an 860 Storm Scanner (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software. All reported values are means ± standard error of the mean (sem).

Results

Hypoxia and acidosis increase cardiac sphingosine levels

An initial experiment was designed to determine if SPH levels increased in ventricular tissue exposed to `hypoxia. Rabbit hearts were used in order to obtain enough ventricular mass for the sphingolipid extraction procedure. A protocol similar to that successfully used by others in the Langendorff model [24] was used in an attempt to recreate the hypoxic/acidotic conditions associated with post-MI ischemia, except that 100% CO_2 was utilized to achieve a more acidic internal pH typical of ischemia [21]. After 5 minutes of hypoxia/acidosis, the ventricles were excised, homogenized and rapidly processed for sphingolipid extraction and HPLC. In the control crude homogenate shown in Figure 1, the content of SPH was 2.11 ± 0.27 nmol/gm and increased
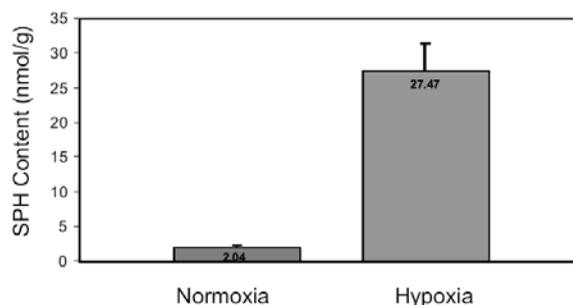

Figure 1. Hypoxia and acidosis increase sphingosine content of Langendorff-perfused rabbit hearts. *Rabbit hearts were perfused for 5 minutes under either normoxic or hypoxic/acidotic conditions as described in Methods. The ventricles were rapidly homogenized in cold buffer and processed for sphingolipid extraction and reverse-phase HPLC. (p < 0.001, Student's T-test; n = 18 normoxia, n = 11 hypoxia).*

significantly (p < 0.0001, Student's T-test) 13-fold to 27.47 ± 3.98 nmol/gm in hypoxic/acidotic conditions.

Hypoxia-triggered apoptosis

The time courses of hypoxia-induced apoptosis and necrosis in adult cardiomyocytes are shown in Figure 2 and demonstrate that at 5 hours of incubation, there was no appreciable cell death; however by 7 hours of hypoxia, significant (~26% or 3.7-fold increase, p < 0.001) apoptosis was evident compared to the normoxic condition. By 12 hours of hypoxia treatment, significant increases in both apoptosis (p < 0.006) and necrosis (p < 0.001) were evident. Many of these cells showed signs of both apoptosis and necrosis. In the absence of tissue macrophages that would otherwise engulf them, many apoptotic cells showed loss of membrane integrity as a sign of secondary necrosis. Control cultures of adult rat cardiomyocytes stained with H/PI demonstrated the typical rod-shaped appearance, crisp myofibril striations and blue-stained elongated nuclei characteristic of healthy cells.

Adult cardiomyocytes released TNFα as a consequence of both the 5 and 7 hour hypoxia treatments. For example during the 7 hours of normoxia, the control (normoxic) adult cardiomyocytes released 810 pg TNFα/10^6 cells (Table 1). Hypoxic cardiomyocytes released significantly (p < 0.001) more cytokine (8660 pg TNFα/10^6 cells, 300 pg/mL, 17.9 nM) into the cell-conditioned media (Table 1).

Sphingosine is produced by hypoxic cardiomyocytes in culture

Table 1 indicates that the cardiomyocytes produced appreciable SPH in response to hypoxia. These selected experiments were designed to determine if SPH was the only sphingolipid released from non-permeabilized, hypoxic cells, adult cardiomyocytes were made hypoxic under conditions that ensured that nearly all of the cells (~98%) in the experiment were viable and not perme-

A

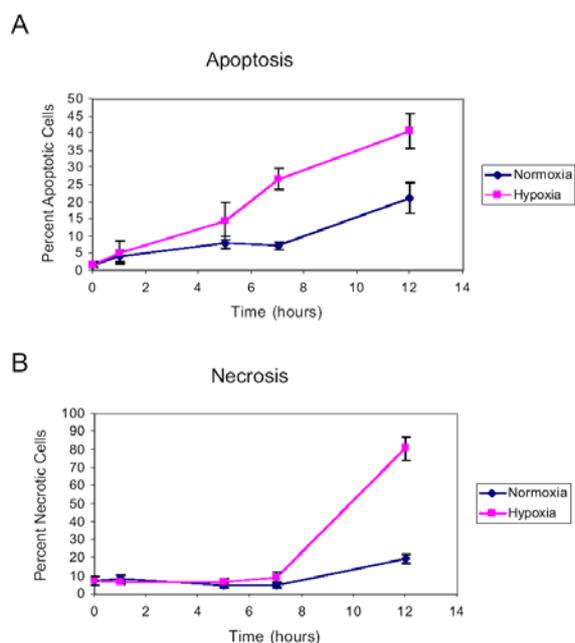### Apoptosis

B

Necrosis

Figure 2. Hypoxia-induced cell death in adult cardiomyocytes. *Adult rat cardiomyocytes were cultured under hypoxic conditions for the indicated times. Cell death by apoptosis was determined by the comet assay (panel A) and necrosis was determined by H/PI (panel B). ANOVA analysis determined that hypoxia produced significant (P < 0.01) time-dependent increase in the percent apoptotic (A) and necrotic (B) dead cells (n = 3).*

abilized to ensure that any observed SPH release came from intact cells. For these experiments, the cultures were washed vigorously to remove H/PI staining red cells prior to treatment so that after 5 hours of normoxia and hypoxia, only $1.44 \pm .19\%$ and $1.83 \pm .15\%$ (n TNFα and Sphingolipid Signaling in Cardiac Hypoxia = 26) permeabilized cells remained in the culture dishes, respectively (Table 1). Importantly, appreciable SPH (20.8 ± 2.7 fold increase, p < .001) was released before an appreciable increase in permeabilization had taken place. The amount of SPH released from the 5-hour normoxic cells increased from 189 ± 24 to 3904 ± 624 pmol/10^6 cells after hypoxic treatment. Comparable

results were obtained from 7 hr incubations (Table 1).

Figure 3A shows typical chromatograms of the cell-conditioned media from both control (normoxic) and hypoxic (95% N_2, 5% CO_2) adult cardiomyocytes. The only sphingolipid base that accumulated was sphingosine (SPH). Figure 3B shows cumulative data from several experiments and demonstrates that hypoxia resulted in the production of substantial amounts of SPH. Importantly, this figure shows that the significant (p < 0.0001) increase in total cell SPH was not reflected in an increased intracellular content, but that nearly all (> 98%) of the SPH produced by hypoxia had been released from the cells into the cell-conditioned media. In addition, the lack of appreciable S1P in cardiomyocytes suggests that these cells lack appreciable sphingosine kinase required to convert SPH to intracellular S1P.

Figure 4 shows the amount of SPH and TNFα (inset) released into the cell-conditioned media and expressed in terms of fold increases associated with hypoxia. These data demonstrate that the SPH response (~20-fold increase over normoxia) was more profound than the TNFα response (~10-fold increase over normoxia, inset). Further, Figure 4 shows that 30 min pretreatment with both the TNFRII:Fc receptor fragment designed to bind free TNFα, and the sphingomyelinase inhibitor, L-carnitine, were capable of significantly (p < 0.01) reducing the amount of released SPH by hypoxic cardiomyocytes.

Released sphingosine is converted to sphingosine-1-phosphate

Rat hearts were excised and perfused with Krebs buffer containing 20 μM SPH (Figure 5). As expected, SPH appeared in the eluate of the hearts. Importantly, none of the Langendorff-perfused hearts demonstrated the presence of appreciable S1P (detection limit is 5 pmol) or other sphingolipids. Figure 5 Panel C shows that a strikingly different result was obtained when fresh blood platelets (see 7×10^5 cells/mL) were added to the perfusate (see Methods). In the presence of platelets, the SPH treated hearts demonstrated appreciable S1P production by 20 minutes as evidenced by the appearance of a very strong S1P peak at its retention time (~16 minutes). By 90 minutes of perfusion, almost all of the SPH had been converted to S1P (Panel D).

Table 1

Time of incubation	5 hrs	5 hrs	7 hrs	7 hrs
Adult cardiomyocytes	Normoxia	Hypoxia	*Normoxia*	*Hypoxia*
% Permeabilized (necrotic) cells	$1.44 \pm .19$ (n=26)	$*1.83 \pm .15$ (n=26)	$1.45 \pm .15$ (n=24)	$*2.11 \pm .18$ (n=30)
TNFα (pg/10^6 cells)	572 ± 25 (n=3)	$*7662 \pm 1985$ (n=6)	810 ± 155 (n=9)	$*8660 \pm 3150$ (n=7)
SPH (pmol/10^6 cells)	189 ± 24 (n=25)	$*3940 \pm 624$ (n=24)	238 ± 39 (n=4)	$*3860 \pm 547$ (n=6)

Values are means ± sem for the indicated number of independent experiments
* Significant from control (respective normoxia) at the p<0.05 level or greater
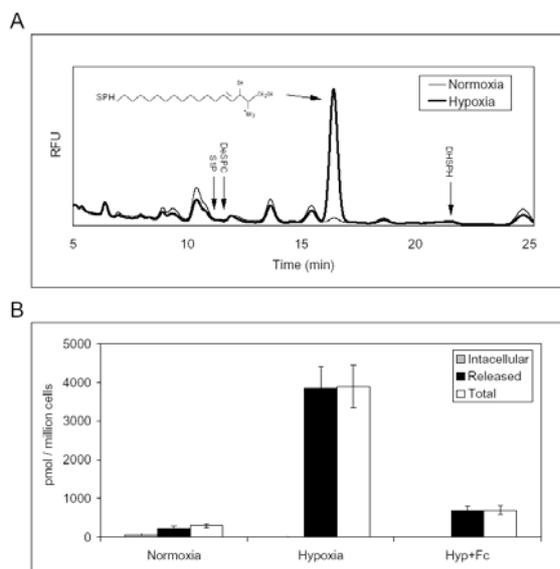
Figure 3. Sphingosine is released from hypoxic cardio-myocytes. *Adult cardiomyocytes were cultured under either normoxia or hypoxia conditions for 5 hours. The cell-conditioned Tyrode's solution was collected and the cells were scraped from the culture dish. Both cellular and extracellular sphingolipids were extracted (see Methods) and then quantified by reverse-phase HPLC (panel A). The retention times of the key sphingolipids (SPH, SPC, S1P, DHSPH) followed in these experiments are shown in panel A. Hypoxia resulted in a substantial increase in a peak corresponding to D-erythrosphingosine (SPH). Panel B shows the cumulative data from 5 separate experiments in which the intracellular and extracellular (released) SPH was determined for hypoxic adult cardiomyocytes. Hypoxia resulted in a significant (p < 0.001) increase in released SPH (n = 19). Over 98% of the total SPH produced by hypoxic cells were released into the culture media. For these cultures, only 2.1 ± 0.2% cells were necrotic (i.e., permeabilized) as a consequence of 5 hours of hypoxia.*

In related experiments, uncoagulated whole blood samples were incubated *in vitro* for up to 48 hours with ^{3}H-SPH and followed by TLC to evaluate the potential conversion of radiolabeled SPH (Figure 6A). The major metabolite that was appreciably labeled was sphingosine-1-phosphate (S1P), presumably by the very active sphingosine kinase in blood [26]. Figure 6B shows cumulative data from several experiments and indicates that the conversion of ^{3}H-SPH to ^{3}H-S1P occurred within 30 minutes and was stable in whole blood for the 15-hour time course of the experiment shown in Figure 5B. Only after several hours was it evident that a small amount of hexadecanal (HD) was produced by the low level of lyase enzyme present in blood platelets capable of converting S1P to hexadecanal and ethanolamine phosphate [26].
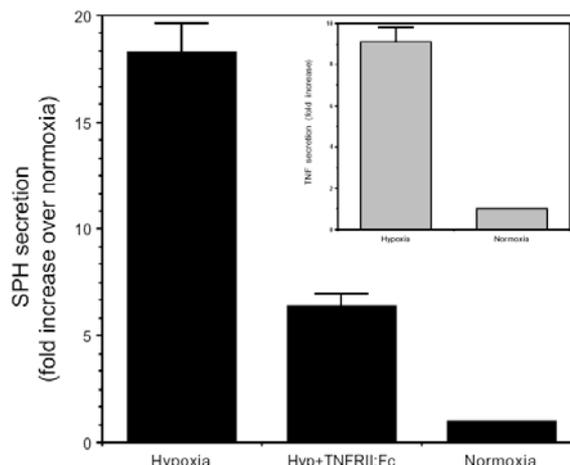

Figure 4. TNFRII:Fc blocks SPH release. *Cumulative data from adult cardiomyocytes treated with hypoxic conditions for 5 hours were compared to normoxic cells also incubated for 5 hours. The fold-increases in SPH and TNFα (inset) in response to hypoxia are shown. Cells were pretreated for 30 min with TNFRII:Fc (0.5 ng/mL) prior to hypoxia (n = 19).*

Discussion

We report here for the first time that cultured cardiomyocytes subjected to hypoxia produce and, importantly, release into the extracellular environment significant quantities of SPH and the pro-inflammatory cytokine, TNFα. The TNFα concentration found in the cell-conditioned media (300 pg/mL, 17.6 nM) exceeds the amount needed to produce its reported negative inotropic effects [4-6] and pro-apoptotic effects [11], even though the cytokine was diluted by the cell culture media to a far greater extent than one would expect *in situ*. SPH also produces negative inotropic and pro-apoptotic cardiac effects [28]. Hypoxia-triggered SPH release is at least in part dependent upon TNFα, since TNFRII:Fc was able to substantially block the effect. During hypoxia, TNFα is likely released before SPH and acts in an autocrine or paracrine manner on TNFα receptors expressed by rat cardiomyocytes [4] to activate sphingomyelinase. Figure 6 shows a hypothetical model of these events.

TNFα triggers cell death in heart cells [11, 27], and anti-TNFα antibodies reduce infarct size in a rabbit coronary occlusion model [28]. However, these data are in apparent conflict with a recent paper showing increased apoptosis in a double TNFRI/RII receptor knock out mouse in the early (3 hrs) but not later post-MI periods [29]. Explanations for the paradoxical actions of TNFα have been recently reviewed [30] and include both time and concentration dependent effects of the cytokine on the heart, as well as the fact that TNFα activates multiple signaling pathways.
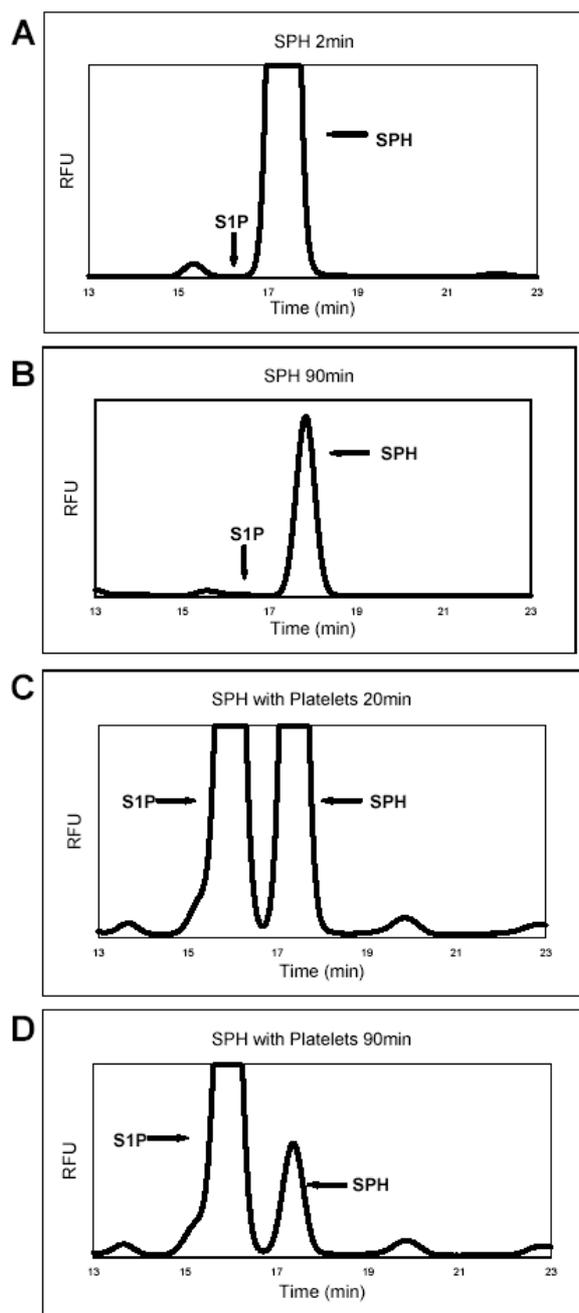
Figure 5. Sphingosine-treated rat hearts do not produce S1P. *Excised rat hearts were perfused with SPH (20 μM) to determine if any cardiac cellular components were capable of metabolizing SPH. Shown in Panels A+B are typical HPLC chromatograms of the eluate produced by a rat heart perfused for 2 min and 90 min, respectively, with Krebs-Henseleit buffer showing only SPH in the eluate even after 90 min of treatment. Panels C + D show an experiment in which the heart was perfused for 20 min and 90 min, respectively, with Krebs-Henseleit containing 7 x 10^5 purified human blood platelets. The gain on these chromatograms was purposefully increased so that in Panels A and B in particular, any S1P present in the eluate would be seen.*
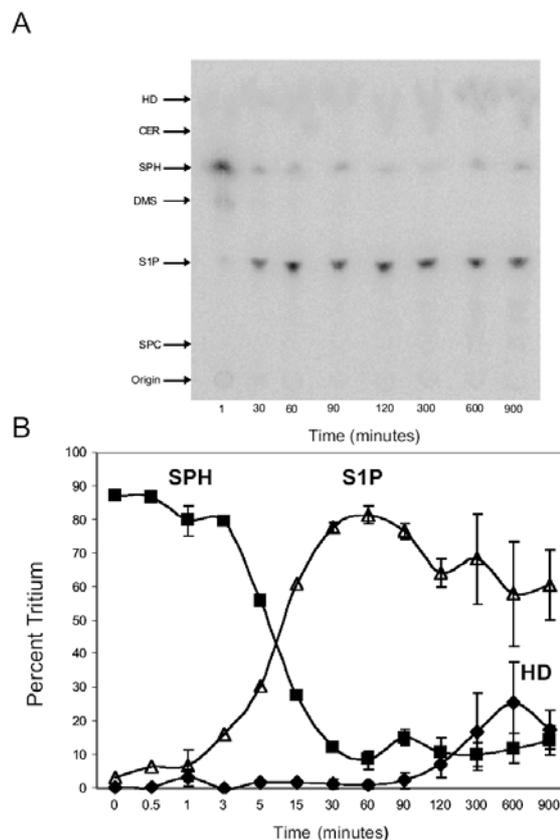

Figure 6. Sphingosine is rapidly converted to sphingosine-1-phosphate by whole blood. *Fresh human whole blood samples were incubated with ^{3}H-SPH for the indicated time periods, followed by butanol extraction and chromatographic separation by TLC (A). This solvent system is capable of separating the major sphingolipids by their Rf values: sphingosylphosphoryl choline (SPC) sphigosine-1-phosphate (S1P), dimethylsphingosine (DMS), D-erythrosphingosine (SPH), ceramide (CER) and hexadecanal (HD). The autoradiogram shows the conversion of SPH into S1P. Panel B shows the results of 5 separate experiments like the one shown in (A). Over the 15 hour (900 minute) time period of the experiment, S1P was nearly the only sphingolipid that was tritium labeled.*

Our finding that SPH is produced by hypoxic cardiomyocytes is novel. Since SPH is rapidly formed from ceramide [34, 35], this is consistent with ceramide production being one of the earliest responses to hypoxia and reoxygenation in cultured cardiomyocytes [14] and a 4.5-fold increase in ceramide content in hypoxic rat neonatal cardiomyocytes [31]. Sphingosine could be produced and released from the *extracellular leaflet* of the membrane where both sphingomyelin and sphingomyelinase are located [32].

From the 3860 pmol SPH released/10^6 cells demonstrated in this study and assuming the adult human has a 250-300 g heart and a 5 L blood volume, one can esti-
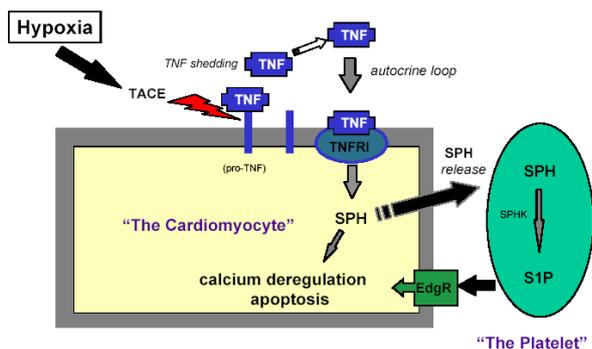
Figure 7. The central role of TNFα and sphingolipids in calcium deregulation and apoptosis in response to hypoxia. *It is hypothesized that one of the earliest responses to hypoxia is the enzymatic processing or "shedding" of membrane-bound TNFα (pro-TNF) by the action of metalomatrix proteases (also known as TACE, or TNFα Converting Enzyme) located in the extracellular matrix of the cardiomyocyte. The TNFα that is released from the cardiomyocyte then acts in an autocrine or paracrine fashion on cardiomyocyte complement of TNFα receptors (TNFRI) that, in turn, activate the sphingomyelin signal transduction system. The principal signaling molecule produced by the TNFα trigger is SPH, the majority of which crosses the sarcolemma membrane and is released into the extracellular fluid compartment. Blood platelets and other blood components that possess sphingosine kinase (SPHK) convert SPH to sphingosine-1-phopshate, S1P. Igarashi and co-workers have shown that S1P is released from platelets and can partition into the serum [24]. Extracellular S1P is then free to act on the cardiomyocyte complement of Edg receptors (EdgR) to exert its negative inotropic and cardiotoxic effects associated with calcium deregulation. SPH and S1P may also contribute to platelet activation and thrombus formation, thus complicating the ischemic episode. Cardiomyocyte-derived TNFα and SPH can be considered extracellular signaling molecules that, in conjunction with S1P, can also signal fibroblasts and endothelial cells (not shown) to undergo post-MI remodeling and angiogenesis, respectively.*

mate that the hypoxic heart may release into the general circulation over ~400 pmol /ml SPH, exceeding the serum SPH levels of healthy human volunteers (87 ± 5 pmol/mL, n=8), in the range of SPH levels (~200 pmol/mL) experienced by patients experiencing ischemia [23] but less than recently reported serum SPH levels of 1,200 pmol/mL in rats subjected to monocrotaline-induced heart failure [13]. The local concentration of SPH in the coronary circulation and cardiac interstitium could be much higher, sufficiently high to reach the micromolar levels of SPH that produce cardio-

toxic and negative inotropic effects. Recently Heusch has demonstrated that canine hearts made ischemic by microembolization showed significant increases in myocardial tissue levels of both TNFα and SPH [15]. Our findings that both mediators are 'released' from ischemic cardiomyocytes suggests that measuring tissue levels may dramatically underestimate the amount of TNFα and SPH produced by the ischemic myocardium.

Figure 5 demonstrates that rat hearts perfused with SPH are not capable of metabolizing it to S1P or other sphingolipids, suggesting that the SPH released from the cardiomyocytes needs to move from the interstitial space into the blood in order to be converted to S1P, presumably by blood components such as platelets [26]. Thus, it is likely that SPH does not have a chance to accumulate once it has contact with the blood and is instead converted to its bioactive metabolite, S1P. The intercellular transfer of SPH between co-cultured CTLL and A31 cells has been demonstrated by others [33]. The protective action afforded by platelets "sponging" SPH and converting it to S1P may have only temporary advantages, however. If the S1P half-life in platelets is relatively long-lived, then platelet activation as at a thrombus would release high local concentrations of S1P that could have other untoward effects.

S1P has only recently been appreciated as an extracellular mediator that may contribute to cardiovascular disease [3, 34]. For example, S1P produces arrhythmias and coronary vasoconstriction [35, 36]. Extracellular S1P may also contribute to the negative inotropic state associated with ischemia. We have recently reported that cardiomyocytes express novel G protein-coupled receptors belonging to the Edg family and that Edg-1 mediates S1P's ability to cause a form of calcium deregulation characterized by increased diastolic calcium (i.e., calcium overload) and reduced systolic calcium (i.e., negative inotropism) [37], similar to the calcium responses exhibited by the ischemic myocardium [38, 39]. Thus, if released from platelets, S1P could contribute to the negative inotropic effects of TNFα. Moreover, S1P may be a potent activator of intravascular thrombus [26, 40], providing a possible positive feedback loop to exacerbate coronary ischemia.

Even though S1P at levels higher than 10 μM cause cell death in neonatal myocytes [41], low S1P levels can actually protect from hypoxic injury [41] and stimulate cardiomyocyte hypertrophy [42]. In addition, we speculate that S1P may act as an extracellular signaling molecule to participate in post-MI remodeling events such as fibroblast proliferation and collagen gene expression and the resulting scar formation. In support of this post-MI remodeling role is the recent finding that S1P is a very potent stimulator of angiogenesis [43]. In our laboratory, S1P stimulates cardiac fibroblast proliferation and collagen I gene expression (unpublished observations). One may consider that all three mediators, TNFα, SPH and S1P, act as extracellular signals for

acute effects and that TNFα and S1P also contribute to compensatory and remodeling events.

In sum, the current study demonstrates that hypoxia generates not only TNFα but also extracellular SPH, the majority of which can be converted to S1P by platelets. This study is the first to provide evidence that TNFα-derived sphingolipid mediators are derived from hypoxic cardiomyocytes themselves. These mediators are important extracellular signaling molecules that participate in the pathology of cardiac ischemia.

Address correspondence to:

Roger A. Sabbadini, Ph.D., Department of Biology, San Diego State University, 5500 Campanile Dr., San Diego, CA 921182-4614, fax (619) 594-0626, Email rsabba@sunstroke.sdsu.edu.

References

[1] Hannun YA, Bell RM: The sphingomyelin cycle: a prototypic sphingolipid signaling pathway. *Adv Lipid Res* 1993; 25: 27-41.

[2] Igarashi Y. Functional roles of sphingosine, sphingosine-1-phosphate, and methylsphingosines: In regard to membrane sphingolipid signaling pathways. *J Biochem* 1997; 122: 1080-1087.

[3] Levade T, Auge N, Jan Veldman R, Cuvillieer O, Negre-Salvayre A, Salvayre R: Sphingolipid mediators in cardiovascular cell biology and pathology. *Circ Res* 2001; 89: 957-968.

[4] Krown K, Yasui K, Brooker M, et al: TNFα receptor expression in rat cardiac myocytes: TNFα inhibition of L-type Ca^{2+} current and Ca^{2+} transients. *FEBS Letters* 1995; 376: 24-30.

[5] Oral H, Dorn GW, Mann DL: Sphingosine mediates the immediate negative inotropic effects of tumor necrosis factor-α in the adult mammalian cardiac myocyte. *J Biol Chem* 1997; 272: 4836-4842.

[6] Sugishita K, Kinugawa K, Shimizu T, et al: Cellular basis for the acute inhibitory effects of Il-6 and TNF-α on excitation-contraction coupling. *J Mol Cell Cardiol* 1999; 31: 1457-1467.

[7] Friedrichs GS, Swillo RE, Jow B, et al: Sphingsine modulates myocyte electrophysiology, induces negative inotropy and decreases survival after myocardial ischemia. *J Cardio Pharm* 2002; 39: 18-28.

[8] Dettbarn C, Betto R, Salviati G, Palade P, Jenkins GM, Sabbadini RA: Modulation of cardiac SR ryanodine receptor by endogenous sphingosine. *J Mol Cell Biol* 1994; 26: 292-342.

[9] McDonough PM, Yasui K, Betto R, et al: Control of cardiac Ca^{2+} levels: inhibitory actions of sphingosine on Ca^{2+} transients and L-channel conductance. *Circ Res* 1994; 75: 981-989.

[10] Yasui K, Palade PT: Sphingolipid action on sodium and calcium currents of rat ventricular myocytes. *Am J Physiol* 1995; 270: C645-9.

[11] Krown KA, Page MT, Nguyen C, et al: TNFα-induced apoptosis in cardiac myocytes: Involvement of the sphingolipid signalling cascade in cardiac cell death. *J Clin Invest* 1996; 98: 2854-2865.

[12] Zechner D, Craig R, Hanford D, McDonough PM, Sabbadini RA, Glembotski CC: MKK6 inhibits myocardial cell apoptosis via a p38 MAP kinase-dependent pathway. *J Biol Chem* 1998; 273: 8232-8239.

[13] Dalla Libera L, Sabbadini R, Renken C, et al: Apoptosis in the skeletal muscle of rats with heart failure is associated with increased serum levels of TNFα and sphingosine. *J Mol Cell Cardiol* 2001; 33: 1871-1878.

[14] Hernandez O, Discher D, Bishorpric N, Webster K: Rapid activation of neutral sphingomyelinase by hypoxia-reoxygenation of cardiac myocytes. *Circ Res* 2000: 198-204.

[15] Thielmann M, Dörge H, Martin C, et al: Myocardial dysfunction with coronary microembolization: signal transduction through a sequence of nitric oxide, tumor necrosis factor-α, and sphingosine. *Circ Res* 2002; 90: 807-813.

[16] Squadrito F, Altavilla D, Zingarelli B, et al: Tumor necrosis factor involvement in myocardial ischemia-reperfusion injury. *Eur J Pharmacol* 1993; 237: 223-230.

[17] Herrmann SM, Ricard S, Nicaud V, et al: Polymorphisms of the tumor necrosis factor-α gene, coronary heart disease and obesity. *Eu J Clin Invest* 1998; 28: 59-66.

[18] Meldrum DR, Cleveland JC, Cain BS, Meng X, Harken AH: Increased myocardial Tumor Necrosis Factor-α in a crystalloid-perfused model of cardiac ischemia-reperfusion injury. *Ann Thorac Surg* 1998; 65: 439-443.

[19] Cain BS, Meldrum DR, Meng X, et al: p38 MAPK inhibition decreases TNF-alpha production and enhances postischemic human myocardial function. *J Surg Res* 1999; 83: 7-12.

[20] Comstock KL, Krown KA, Page MT, et al: LPS-induced TNF-α release from and apoptosis in rat cardiomyocytes: obligatory role for CD14 in mediating the LPS response. *J Mol Cell Cardiol* 1998; 30: 2761-2775.

[21] Yokoyama T, Sekiguchi K, Tanaka T, et al: Angiotensin II and mechanical stretch induce production of tumor necrosis factor in cardiac fibroblasts. *Am J Physiol* 1999; 276: H1968-H1976.

[22] Torre-Amione G, Kapadia S, Lee J, Bies RD, Lebovitz R, Mann DL: Expression and functional significance of tumor necrosis factor receptors in human myocardium. *Circulation* 1995; 92: 1487-1493.

[23] Deutschman D, Carstens J, Klepper R, et al: Predicting obstructive coronary artery disease using serum sphingosine-1-phosphate. *Am Heart J*

serum sphingosine-1-phosphate. *Am Heart J* 2002; Submitted: Submitted.

[24] Page MT, Quintana PJE, Ligutti JA, Sabbadini RA: Cell death in cultured adult rat cardiomyocytes: use of the comet assay to distinguish apoptosis from necrosis. *Basic Appl Myol* 2000; 10: 159-170.

[25] Caligan TB, Peters K, Ou J, Wang E, Saba J, Merrill AH: A high-performance liquid chromatographic method to measure sphingosine 1-phosphate and related compounds from sphingosine kinase assays and other biological samples. *Anal Biochem* 2000; 281: 36-44.

[26] Yatomi Y, Igarashi Y, Yang L, et al: Sphingosine-1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J Biochem* 1997; 121: 969-973.

[27] Song W, Lu X, Feng Q: Tumor necrosis factor-α induces apoptosis via inducible nitric oxide synthase in neonatal mouse cardiomyocytes. *Cardio Res* 2000; 45: 595-602.

[28] Li D, Zhao L, Liu M, et al: Kinetics of tumor necrosis factor α in plasma and the cardioprotective effect of a monoclonal antibody to tumor necrosis factor α in acute myocardial infarction. *Am Heart J* 1999; 137: 1145-1152.

[29] Kurrelmeyer KM, Michael LH, Baumgarten G, et al: Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine modle of acute myocardial infarction. *PNAS* 2000; 97: 5456-5461.

[30] Sack MN, Smith RM, Opie LH: Tumor necrosis factor in myocardial hypertrophy and ischaemia - an anti-apoptotic perspective. *Cardio Res* 2000; 45: 688-695.

[31] Bielawska AE, Shapiro JP, Jiang L, et al: Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion. *Am J Pathol* 1997; 151: 1257-1263.

[32] Mohan Das DV, Cook HW, Spence MW: Evidence that neutral sphingomyelinase of cultured murine neuroblastoma cells is oriented externally on the plasma membrane. *Biochim Biophys Acta* 1984; 777: 339-342.

[33] Inooka S, Tokokuni T: Sphingosine transfer in cell to cell interaction. *Biochem Biophys Res Com* 1996; 218: 872-876.

[34] Siess W, Essler M, Brandl R: Lysophosphatidic acid and sphingosine 1-phosphate: two lipid villains provoking cardiovascular diseases. *IUBMB Life* 2000; 49: 167-171.

[35] Lilom K, Sun G, Bunemanns M, et al: Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors. *Biochem J* 2001; 355: 189-197.

[36] Sugiyama A, Yatomi Y, Ozaki Y, Hashimoto K: Sphingosine 1-phosphate induces sinus tachycardia and coronary vasoconstriction in the canine heart. *Cardiovasc Res* 2000; 46: 119-125.

[37] Nakajima N, Cavalli AL, Biral D, et al: Expression and characterization of Edg-1 receptors in rat cardiomyocytes: Calcium deregulation in response to sphingosine-1-phosphate. *Eur J Biochem* 2000; 267: 5679-5686.

[38] Lee H, Mohabir R, Smith N, Franz M, Clusin WT: Effect of ischemia on calcium-dependent fluorescence transients in rabbit hearts containing Indo-1. *Circ* 1988; 78: 1047-1059.

[39] Kihara Y, Grossman W, Morgan JP: Direct measurement of changes in intracellular calcium transients during hypoxia, ischemia, and reperfusion of the intact mammalian heart. *Circ Res* 1989; 65: 4: 1029-1044.

[40] Yatomi Y, Ruan F, Hakomori S, Igarashi Y: Sphingosine-1-phosphate: a platelet-active sphingolipid released from agonist-stimulated human platelets. *Blood* 1995; 86: 193-202.

[41] Karliner JS, Honbo N, Summers K, Gray MO, Goetzl EJ: The lysophospholipids sphingosine-1-phosphate and lysophosphatidic acid enhance survival during hypoxia in neonatal rat cardiac myocytes. *J Mol Cell Cardiol* 2001; 33: 1713-1717.

[42] Robert P, Tsui P, Lavill MP, Sarau HM, Bril A, Berrebi-Bertrand I: EDG1 receptor stimulation leads to cardiac hypertrophy in rat neonatal myocytes. *J Mol Cell Cardiol* 2001; 33: 1589-1606.

[43] Lee MJ, Thangada S, Claffey KP, et al: Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell* 1999; 99: 301-12.