Basic Res Cardiol 96: 267 – 274 (2001)
© Steinkopff Verlag 2001

ORIGINAL CONTRIBUTION

David X. Zhang
Ryan M. Fryer
Anna K. Hsu
Ai-Ping Zou
Garrett J. Gross
William B. Campbell
Pin-Lan Li

Production and metabolism of ceramide in normal and ischemic-reperfused myocardium of rats

Received: 4 October 2000
Returned for revision: 23 October 2000
Revision received: 22 November 2000
Accepted: 5 December 2000

D. X. Zhang · R. M. Fryer · A. K. Hsu
A.-P. Zou · G. J. Gross, Ph.D. (✉)
W. B. Campbell · P.-L. Li
Department of Pharmacology
and Toxicology
Medical College of Wisconsin
8701 Watertown Plank Rd
Milwaukee, WI 53226, USA
E-mail: ggross@mcw.edu

Abstract Ceramide has been shown to be a key signaling molecule involved in the apoptotic effect of tumor necrosis factor α (TNF-α) and other cytokines. Given the importance of cytokines such as TNF-α in myocardial ischemia-reperfusion injury, we hypothesize that ceramide is increased during ischemia or reperfusion, and that the activity of enzymes responsible for its production or breakdown should be increased and/or decreased, respectively. Therefore, in the present study, we characterized the enzymatic activities responsible for ceramide production and metabolism in the myocardium of rats, and determined the contribution of these enzymes to altered ceramide levels during myocardial ischemia and reperfusion. The basal ceramide concentration in the myocardium of rats was 34.0 pmol/mg tissue. As determined by the conversion of ^{14}C-sphingomyelin into ceramide and ^{14}C-choline phosphate, both neutral (N-) and acidic (A-) SMase were detected in the myocardium, with a conversion rate of 0.09 ± 0.008 and 0.32 ± 0.05 nmol/min per mg protein, respectively. The activity of A-SMase (78 % of total cellular activity) was significantly higher in microsomes than in cytosol, while the activity of N-SMase was similar in both fractions. Ceramidase, a ceramide-metabolizing enzyme, was also detected in the myocardium of rats. It metabolized ceramide into sphingosine at a rate of 9.94 ± 0.42 pmol/min per mg protein. In anesthetized rats, 30 min of ischemia had no apparent effect on ceramide concentrations in the myocardium, while 30 min of ischemia followed by 3 h of reperfusion resulted in a significant increase in ceramide by 48 %. The activities of both N- and A-SMase were reduced by 44 % and 32 %, respectively, in the myocardium subjected to ischemia followed by reperfusion, but unaltered in the ischemic myocardium. It was also found that myocardial ischemia followed by reperfusion produced a marked inhibition of ceramidase (by 29 %). These results demonstrate that the myocardium of rats expresses N- and A-SMase and ceramidase, which contribute to the production and metabolism of ceramide, respectively. Tissue ceramide concentrations increased in reperfused myocardium. These increases in ceramide were not associated with enhanced SMase activity, but rather with reduced ceramidase activity.

Key words Ceramide – sphingomyelinase – ceramidase – ischemia – reperfusion – heart – rats

BRC 262

© Steinkopff Verlag 2001

Introduction

The mechanisms of myocardial ischemic-reperfusion injury have been extensively studied. Various cytokines such as TNF-α may mediate or promote myocardial ischemic-reperfusion injury (3, 8–10, 24, 25). However, the cellular mechanisms mediating the action of these cytokines in myocardial injury are poorly understood. Recent studies have indicated that the sphingolipid metabolites are involved in the reduction of myocardial contractility during ischemia and reperfusion (28). It has been demonstrated that ceramide, a sphingolipid second messenger, mediates the actions of different cytokines such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interferon-γ (12, 13, 18, 22). Therefore, ceramide may play a role in mediating physiological or pathological response to cytokines. Based on these observations, we hypothesize that ceramide is increased during ischemia or reperfusion, and that increased ceramide may mediate the detrimental effect of TNF-α or other cytokines, resulting in myocardial injury.

Ceramide is produced primarily by the hydrolysis of membrane sphingomyelin (SM) through sphingomyelinase (SMase), or from pamitoyl CoA and serine through *de novo* synthesis. Ceramide, once generated, can be metabolized or converted to other molecules by various enzymes such as ceramidase, glucosylceramide synthase, ceramide kinase, and SM synthase (13, 22). With respect to the alteration of intracellular ceramide in response to cytokines, it has been demonstrated that this alteration mainly resulted from the modulation of two isoforms of SMase, the neutral (N-) and acidic (A-) SMase, and ceramidase in a variety of mammalian tissues and cells. A- and N-SMase were found to be stimulated by TNF-α, IL-1β, and interferon-γ, and ceramidase can be activated or inhibited by these cytokines (6, 12, 17, 18, 23, 26). Other enzymes involved in ceramide production or metabolism are either not well characterized or not associated with the effects of cytokines (13, 22). The present study was designed to determine the tissue levels of ceramide in normal and ischemic/reperfused rat myocardium. Then, we went on to explore the mechanisms involved in altered ceramide concentrations in the myocardium subjected to ischemia and/or reperfusion. Given that SMase and ceramidase represent the major enzymes responsible for cytokine-induced changes in ceramide, we determined the contribution of these two enzymatic pathways to changes in ceramide levels in the myocardium during ischemia and reperfusion.

Methods

Ceramide assay

Ceramide was determined by a modification of the method of Preiss et al (29). In brief, frozen myocardial tissues (50 mg) were homogenized in 4 volumes of 10 mM phosphate buffered saline (PBS), and the lipids were extracted, dried under N2 and used for the analysis of ceramide within 72 h. An aliquot of dried lipid (1–2 mg tissue) was solubilized by bath sonication into a detergent solution (7.5 % n-octyl-b-D-glucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriaminepentaacetic acid (DTPA), and mixed with bacterial DG kinase (Calbiochem, CA) and 4 µCi [γ-^{32}P] ATP to a final volume of 100 µl. After incubating at 25 °C for 3 h, the reaction was stopped by extraction of lipids with 600 µl chloroform:methanol (1:1, v/v), 20 µl 1 % perchloric acid and 150 µl 1 M NaCl. Then, the lower organic phase was recovered, washed twice with 1 % perchloric acid and dried with N_2. The ^{32}P-labeled ceramide was separated from other lipids by thin layer chromatography (TLC) with a solvent consisting of chloroform:acetone: methanol:acetic acid:water (10:4:3:2:1, v/v/v). After visualization by autoradiography, the ceramide 1-^{32}P band was recovered by scraping and counted in an Beckman liquid scintillation counter. The amount of ceramide in the lipid extracts was calculated from a standard curve constructed with known amounts of C_{16} ceramide, and expressed as pmol/mg tissue. To assure that detected differences in ceramide concentrations during ischemia and reperfusion were not due to effects of different tissue samples on the activity of the DAG kinase in the *in vitro* assay, the phosphorylation of C_2-ceramide as an internal standard was determined in parallel. This assay was validated by repeated measurements using the homogenate from the same heart. The coefficient variation (C.V.) was 5.3 %.

Preparation of homogenates, cytosol and microsomes from rat myocardium

The left ventricles freshly dissected from male adult Wistar rats (350–450 g) were minced, washed twice with PBS, and homogenized in ice-cold HEPES buffer containing (in mM): Na-HEPES, 25; sucrose, 255; EDTA, 1; and phenylmethylsulfonyl fluoride, 0.1 (pH 7.4). After centrifugation at 1,000 x g for 10 min at 4 °C, the supernatants containing the membrane protein and cytosolic components, termed homogenates, were frozen in liquid N_2, and stored at –80 °C until used. Microsomal and cytosolic fractions were prepared by a sequential centrifugation of the homogenate at 10,000 x g for 20 min and 100,000 x g for 90 min as described previously (20).

Total protein concentrations of the sample were measured by the Bio-Rad Protein Assay.

Sphingomyelinase activity assay

The activity of SMase was determined as reported by Liu and Hannun (21). Briefly, [N-methyl ^{14}C]-SM was incubated with myocardial homogenate, cytosol or microsomes, and the metabolites of SMase, ^{14}C-choline phosphate and ceramide were determined. For N-SMase, an aliquot of samples (100 μg protein unless otherwise indicated) was incubated with a neutral assay mixture containing 100 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.05 % Triton X-100, and 0.075 mM [N-methyl ^{14}C]-SM (0.02 μCi) in a final volume of 100 μl. After incubation at 37 °C for 15 min, the reaction was stopped by adding 1.5 ml of chloroform/methanol (2:1, v/v), followed by addition of 0.2 ml of H_2O. The samples were then vortexed and centrifuged at 3,000 rpm for 5 min to separate the two phases. A portion of the upper aqueous phase was transferred to scintillation vials and counted for the formation of ^{14}C-choline phosphate in a Beckman liquid scintillation counter. The activity of A-SMase was determined according to the same protocols as described above, except an acidic assay mixture containing 100 mM sodium acetate, pH 5.0, 0.05 % Triton X-100, and 0.1 mM [^{14}C]-SM (0.02 μCi) was used.

To confirm the accumulation of ceramide in the reaction mixtures, TLC was performed as described previously (19). Briefly, the lower organic phase after lipid extraction was collected and separated on a silica gel TLC LK6D plate (Whatman, NJ) with two solvent systems (chloroform/methanol/25 % ammonium hydroxide/water 50:50:2:1 and 90:10:0.5:0.5, v/v/v/v). SM and ceramide were visualized by iodine staining, and compared with synthetic SM and C_{18} ceramide standards.

Ceramidase activity assay

The activity of ceramidase was measured by the method of Bielawshka et al (1). The assay mixture contained 0.05 mM N-[^{14}C-palmitoyl]-sphingosine (C_{16} ceramide, 0.02 μCi), 0.25 mg Triton-X-100, 0.4 mg sodium cholate, 0.125 M HEPES (pH 8.0) buffer, 0.125 M sucrose, 0.05 mM EDTA, and 200 μg protein in a final volume of 0.2 ml. Incubation was at 37 °C for 2 h. At the end of incubation, 10 μl of 10 mg/ml ice-cold palmitic acid was added to serve as a carrier. The ^{14}C-palmitic acid was separated by the addition of 2 ml of basic Dole's solution (isopropanol : heptane : 1 N NaOH, 40 : 10 : 1, v/v/v), 1.2 ml of heptane, and 1.0 ml of H_2O. After vortexing and centrifugation, the upper phase was discarded and the lower phase was washed twice with 1 ml of heptane and the upper phase was discarded each time. Then 1 ml of 1 N H_2SO_4 and 2 ml of heptane were added. After vortexing and centrifugation, 1 ml aliquots from the upper phase were analyzed for the formation of ^{14}C-palmitic acid by a Beckman liquid scintillation counter.

In vivo myocardial ischemia/reperfusion

Myocardial ischemia/reperfusion was produced as described previously (31). The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Briefly, male Wistar rats weighing 350–450 g were anesthetized by Inactin (100 mg/kg, ip). A tracheotomy was performed, and the rat was intubated with a cannula connected to a rodent ventilator. Body temperature was monitored and maintained at 37 ± 1 °C using a heating pad. The right carotid artery was cannulated to measure blood pressure and heart rate. A left thoracotomy was performed ~10 mm from the sternum to expose the heart at the fifth intercostal space. The pericardium was removed, and the left atrial appendage was moved to reveal the location of the left coronary artery. A ligature (6-0 prolene), along with a snare occluder, was placed around the vein and left coronary artery close to the place of origin. After surgical preparation, the rat was allowed to stabilize for 15 min before the various interventions.

Rats were randomly assigned to the following three groups: group I with no ischemia, group II with 30 min occlusion without reperfusion, group III with 30 min occlusion followed by 3 h reperfusion. After each experiment, the left coronary artery was reoccluded, and a Patent blue dye was injected intravenously to stain the nonischemic area of the left ventricle. The unstained area represented ischemic or "necrotic tissue". After rapid removal of the heart, the ischemic and nonischemic tissue of the left ventricle were isolated, rinsed, and frozen in liquid nitrogen for the measurement of ceramide and the activities of both SMase and ceramidase.

Statistical analysis

Data are presented as mean ± SEM. Significant differences in mean values within and between multiple groups was examined with ANOVA for repeated measures followed by Duncan's post hoc test. $P < 0.05$ was considered statistically significant.

Basic Research in Cardiology, Vol. 96, No. 3 (2001)
© Steinkopff Verlag 2001

Results

Ceramide concentrations in normal rat myocardium

To determine the basal levels of ceramide in the rat myocardium, 6 rat hearts without experimental intervention were used. It was found that the basal ceramide concentration in normal rat myocardium was 34.0 ± 2.3 pmol/mg tissue, and that the detected ceramide concentrations were in proportion to the amount of tissue used. In these experiments, different amounts of tissue obtained by a serial dilution were used to quantitate ceramide. Ceramide was detected in 1 mg of myocardial tissue.

Activity of N- and A-SMase in homogenates, cytosol, and microsomes prepared from normal rat myocardium

As shown in Fig. 1A, a protein concentration-dependent increase in the activity of both N- and A-SMase was detected in rat myocardial homogenates. The C.V. for the N- and A-SMase assay were 2.8 % and 2.1 %, respectively. The activity of A-SMase was 0.32 ± 0.05 nmol/min per mg protein, which was significantly greater than that of N-SMase (0.09 ± 0.008 nmol/min per mg protein). A-SMase accounted for 78 % of the total activity in myocardial homogenates. Comparison of subcellular fractions prepared from rat myocardium revealed the presence of a significantly higher amount of A-SMase in microsomes compared to the cytosol. In contrast, no significant difference between microsomes and cytosol was observed for N-SMase (Fig. 1B). With the use of TLC, SM and its lipid metabolites, ceramide and sphingosine, were identified. When the homogenate was incubated with [^{14}C] SM, the lipid metabolites had a pattern of migration on TLC similar to that with purified SMase plus [^{14}C]-SM. The bands with Rf of 0.10, 0.60, and 0.82 comigrated with standard SM, sphingosine, and C_{18}-ceramide, respectively (data not shown). To further characterize these two enzymes in the myocardium, a kinetic study of the enzymes was conducted. Figure 2 shows the dependence of N- and A-SMase activity on the substrate concentra-

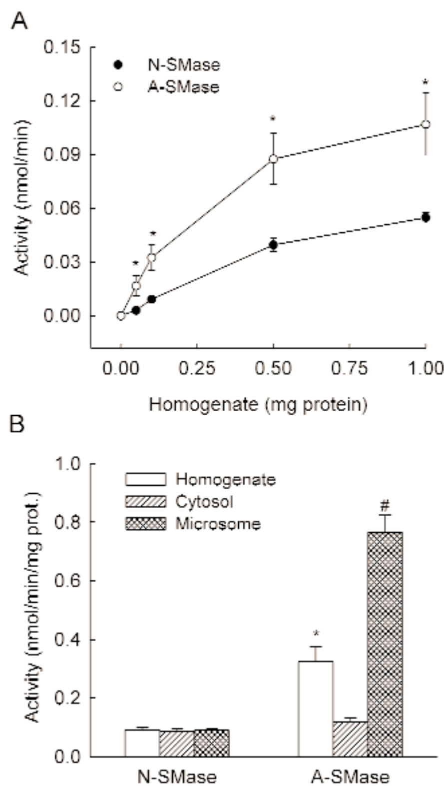

Fig. 1 Activity of N- and A-SMase in the rat myocardium. **A** Relationship of N- and A-SMase activity to protein concentrations in the homogenates. The indicated amount of protein was mixed with 0.075 (for N-SMase) or 0.1 mM (for A-SMase) [N-methyl ^{14}C]-SM (0.02 µCi) and incubated at 37 °C for 15 min. **B** Comparison of N- and A-SMase activity in the cytosolic and microsomal fractions. n = 5 rats, duplicate assay. * P < 0.05 vs N-SMase. # P < 0.05 vs cytosols.

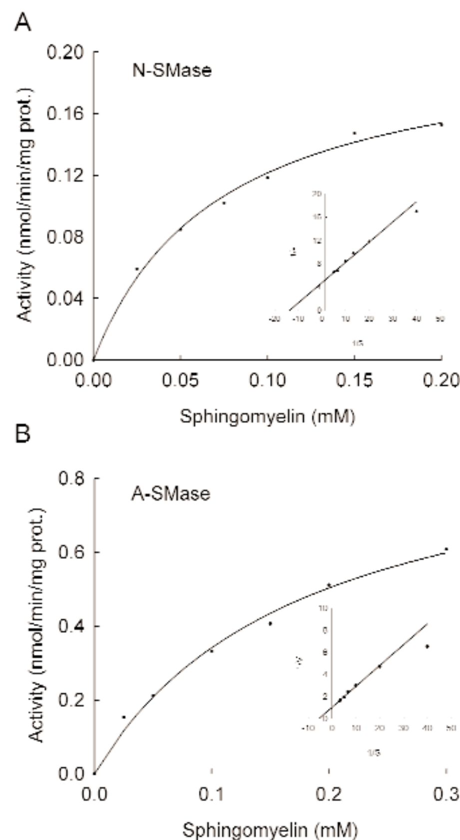

Fig. 2 Kinetic analyses of N- (**A**) and A-SMase (**B**) in the rat myocardium. The indicated concentrations of unlabeled SM and 0.02 µCi [^{14}C]-SM were incubated with the myocardial homogenates at 37 °C for 15 min. Lineweaver-Burk plot of the original data was drawn for the calculation of Km and Vmax values. n = 5 rats, duplicate assay.

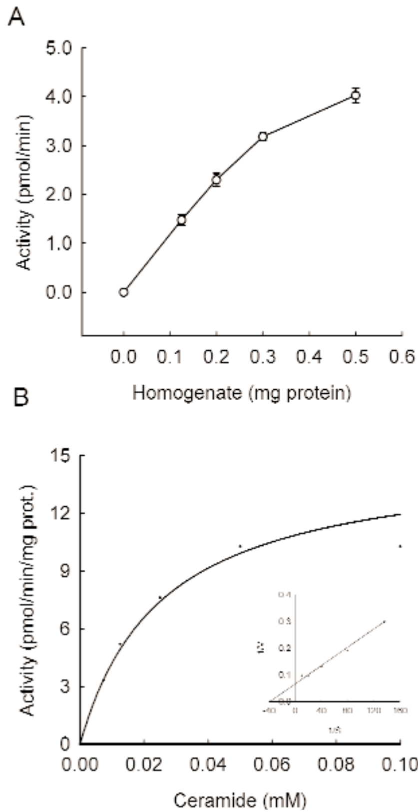

Fig. 3 Activity of ceramidase in the rat myocardium. **A** Relationship of ceramidase activity to protein concentrations in the homogenates. The indicated amount of protein was mixed with 0.05 mM N-[^{14}C-palmitoyl]-sphingosine (0.02 μCi) and incubated at 37 °C for 120 min. **B** Kinetic analysis of ceramidase. The indicated concentrations of unlabeled and 0.02 μCi N-[^{14}C-palmitoyl]-sphingosine were incubated with the myocardial homogenates at 37 °C for 120 min. Lineweaver-Burk plot of the original data was drawn for the calculation of Km and Vmax values. n = 5 rats, duplicate assay.

tions. The Km and Vmax values for N- and A-SMase were 0.07 and 0.18 mM, 0.21 and 0.96 nmol/min per mg protein, respectively.

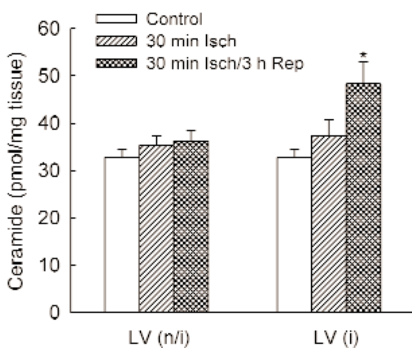

Fig. 4 Concentrations of ceramide in the rat myocardium during *in vivo* ischemia and reperfusion. The concentrations of ceramide were measured in the ischemic (i) and nonischemic (n/i) areas of left ventricle (LV). n = 6 rats, duplicate assay. * $P < 0.05$ vs control.

Activity of ceramidase in normal rat myocardium

As shown in Fig. 3A, a protein concentration-dependent increase in the activity of ceramidase was detected in rat myocardial homogenates. The C.V. for the assay was 4.9 %. The activity of ceramidase was 9.94 ± 0.42 pmol/min per mg protein. TLC analysis of the reaction mixture confirmed that ceramide was hydrolyzed to produce free fatty acids (data not shown). A kinetic study of this enzyme indicated a Km value of 0.025 mM, and a Vmax of 15.0 pmol/min per mg protein (Fig. 3B)

Concentrations of ceramide in rat myocardium during in vivo ischemia and reperfusion

The effect of ischemia and reperfusion on ceramide concentrations in the myocardium is shown in Fig. 4. Thirty minutes of ischemia had no significant effect on ceramide in either ischemic or nonischemic myocardial tissue. However, 3 h of reperfusion led to a 48 % increase in tissue ceramide in the previously ischemic area. There was no significant change in ceramide concentrations in nonischemic tissue from the same heart.

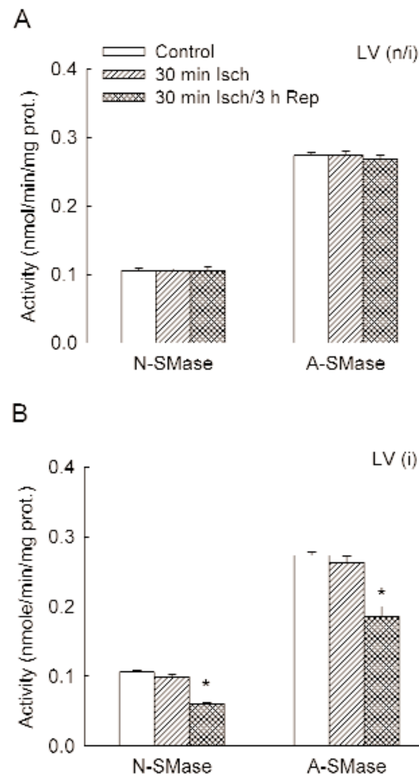

Fig. 5 Activity of N- and A-SMase in the rat myocardium during *in vivo* ischemia and reperfusion. The activity of SMase was measured in the non-ischemic (n/i) (**A**) and ischemic area (i) (**B**) of left ventricle (LV). n = 6 rats, duplicate assay. *$P < 0.05$ vs control.

Basic Research in Cardiology, Vol. 96, No. 3 (2001)
© Steinkopff Verlag 2001

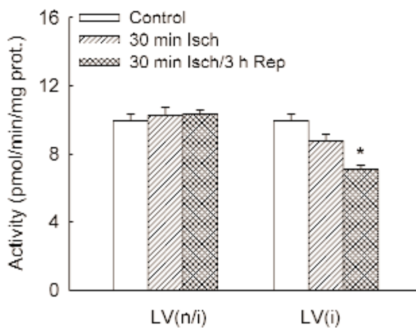

Fig. 6 Activity of ceramidase in the rat myocardium during *in vivo* ischemia and reperfusion. The activity of ceramidase was measured in the non-ischemic (n/i) and ischemic area (i) of left ventricle (LV). n = 6 rats, duplicate assay. *$P < 0.05$ vs control.

Activity of N- and A-SMase in rat myocardium during *in vivo* ischemia and reperfusion

To determine whether the increase in tissue ceramide concentrations during reperfusion is associated with increased production via N- or A-SMase, we measured the activity of both enzymes in the myocardium subjected to ischemia and/or reperfusion. As shown in Fig. 5B, 30 min of ischemia had no significant effect on the activities of N- and A-SMase. However, 30 min of ischemia followed by 3 h reperfusion paradoxically produced significant reductions in both N- and A-SMase activity (by 44 % and 32 %, respectively). There was no change in the activity of either enzyme in non-ischemic tissue (Fig. 5A).

Activity of ceramidase in rat myocardium during *in vivo* ischemia and reperfusion

To determine whether an increase in tissue ceramide concentration during reperfusion is related to decreased metabolism of ceramide, we measured the activity of ceramidase in the myocardium subjected to ischemia and/or reperfusion. As shown in Fig. 6, 30 min ischemia had no significant effect on the activity of ceramidase, while 30 min ischemia followed by 3 h reperfusion resulted in significant decrease in ceramidase activity (by 29 %). There was no apparent alteration in ceramidase activity in the control nonischemic tissue from the same heart.

Discussion

In the present study, basal ceramide was detected in the myocardium of rats, which is most likely the result of the conversion of SM via SMase. A-SMase and N-SMase contributed to 78 % and 28 % of total SMase activity in myocardial tissue, respectively. The activity of A-SMase was significantly higher in the microsomal fraction than in the cytosolic fraction, while the activity of N-SMase was similar in both fractions, suggesting that A-SMase is primarily present on the cell membrane. These results provide the direct evidence that both N- and A-SMase are present in rat myocardial tissue. Both enzymes convert SM into ceramide and choline phosphate.

Several isoforms of SMases have been identified in mammalian cells and tissues. Among these isoforms, A-SMase and N-SMase have been implicated in the hydrolysis of membrane SM to ceramide in response to a variety of cytokines or hormones (7, 17, 23, 27). The greater expression of A-SMase in the myocardial tissue compared with N-SMase is consistent with results found in vascular tissues (19). The Km values of these enzymes in the myocardium are generally in agreement with those of partially purified enzymes (30).

The present study also determined the activity of the ceramide-metabolizing enzyme, ceramidase, in the myocardium of rats. We found that myocardial tissue expressed ceramidase, a membrane-bound enzyme (22). The Km of this enzyme was found to be consistent with those in other tissues (32). Since a number of extracellular stimuli such as TNF-α and IL-1β have been reported to modulate (i.e., down-regulate) the activity of ceramidase (6, 15, 26), the presence of ceramidase in the myocardium may represent another important mechanism in the regulation of intracellular ceramide concentrations.

Previous studies have demonstrated that myocardial ischemia and reperfusion increases tissue cytokines such as TNF-α and IL-1β in the myocardium, which may contribute to depressed cardiac contractility, myocyte apoptosis, and myocardial structural abnormality (3, 24). Since ceramide has been shown to mediate the actions of TNF-α and other cytokines in cell injury (12, 13, 18, 22), it is possible that the over-production of cytokines increases myocardial ceramide concentrations via the modulation of ceramide production or metabolism, thus, leading to myocardial injury during myocardial ischemia and reperfusion.

To explore the role of this ceramide-mediated signaling pathway in myocardial ischemic-reperfusion injury, we examined the levels of ceramide in the ischemic/reperfused myocardial tissue using an *in vivo* ischemia-reperfusion model in rats. Ceramide has been shown to accumulate in the primary cultures of neonatal rat cardiac myocytes during hypoxia and reoxygenation, and in isolated and perfused rat heart under ischemia and reperfusion (5, 14). However, little is known regarding the changes in ceramide levels *in vivo* following ischemia and reperfusion. In the present study, we found that myocardial ischemia did not alter tissue ceramide levels. Myocardial ischemia followed by reperfusion, however, significantly increased tissue ceramide concentrations.

The increase in ceramide concentrations may importantly contribute to myocardial injury during ischemia and reperfusion. Since the accumulation of intracellular ceramide has been implicated in the induction of apoptosis in a number of cell types (4, 11, 16, 18), the effect of ceramide in mediating myocardial ischemia-reperfusion injury may be associated with the induction of cardiomyocyte apoptosis. This view is supported by the finding that exogenous cell-permeable ceramide induced cardiomyocyte apoptosis *in vitro* (2). However, the involvement of some other ceramide-derived signaling molecules could not be excluded. It has been reported that ceramide-derived sphingosine induced negative inotropic effects in the adult mammalian cardiac myocytes by inhibition of intracellular Ca^{2+} mobilization (28).

In the present study, we found that intracellular ceramide was elevated without a concomitant increase in the activity of N- or A-SMase during myocardial ischemia/reperfusion. Ischemia followed by reperfusion induced a significant decrease in the activities of N- and A-SMase. These indicated that the increase in tissue ceramide is not associated with enhanced activity of N- and A-SMase under these circumstances. In the cultured rat cardiac myocytes, hypoxia and reoxygenation has been recently reported to induce the activation of N-SMase and then the accumulation of ceramide (14). The reason for this discrepancy is unknown. It is possible that *in vitro* hypoxia and reoxygenation may be different from the *in vivo* condition of ischemia and reperfusion used in the present study. Recently, Zager et al also reported that ceramide increased without enhancement of SMase activity in ischemic-reperfused kidney tissue. They found that ischemia induced an abrupt decline (~50 %) in SMase activity, which persisted in an unremitting fashion throughout 24 hours of reperfusion (33). These results suggest that ceramide accumulation is not necessarily associated with increased SMase activity, at least in myocardial ischemia and reperfusion.

To further explore other possible mechanisms for the increase in ceramide concentrations during myocardial ischemia and reperfusion, we determined the activity of ceramidase in ischemic-reperfused myocardial tissue. It was found that ischemia followed by reperfusion significantly reduced the activity of ceramidase in ischemic-reperfused tissues, suggesting that inhibition of ceramidase and consequent decrease in ceramide metabolism may be responsible for the increase of intracellular ceramide during myocardial ischemia and reperfusion. Recent studies have indicated that inhibtion of ceramidase by extracellular stimuli contributes to ceramide increase in a number of mammalian tissues or cells (15, 26). Taken together, our results indicated that inhibition of ceramidase, rather than increase in SMase, may be responsible for the accumulation of ceramide during myocardial ischemia followed by reperfusion.

The mechanism responsible for reduction of ceramidase activity during ischemia and reperfusion remains unknown. It should be noted that the decrease in the activity of ceramidase in ischemic-reperfused myocardium may not be associated with nonspecific enzyme destruction, since the activity of other cellular enzymes such as 5'-nucleotidase, ADP-ribosylcyclase and cADP-ribose hydrolase we measured were not altered in the ischemic-reperfused myocardium. It remains to be determined whether reduction of ceramidase in ischemic-reperfused myocardium is associated with cytokine-induced inactivation of this enzyme.

In summary, the present results demonstrate that N-SMase, A-SMase and ceramidase are present in the myocardium of rats. Increases in tissue ceramide levels during ischemia followed by reperfusion are not associated with enhanced SMase activity, but rather with inhibition of ceramidase. This accumulation of ceramide in the reperfused myocardium associated with ceramidase inhibition may play an important role in myocardial injury.

Acknowledgments This study was supported by grants from the National Institutes of Health (HL57244, PLL; HL51055, WBC; HL08311, GJG). The authors thank Gretchen Barg for secretarial assistance.

References

1. Bielawshka A, Greenberg MS, Perry D, Jayadev S, Shayman JA, McKay C, Hannun YA (1996) (1S,2R)-D-eryghro-2-(N-myristoylamino)-1-phenyl-1-propanol as an inhibitor of ceramidase. J Biol Chem 271: 12646–12654
2. Bielawska AE, Shapiro JP, Jiang L, Melkonyan HS, Piot C, Wolfe CL, Tomei LD, Hannun YA, Umansky SR (1997) Ceramide is involved in triggering of cardiomyocyte apoptosis induced by ischemia and reperfusion. Am J Pathol 151: 1257–1263
3. Cain BS, Harken AH, Meldrum DR (1999) Therapeutic strategies to reduce TNF-α mediated cardiac contractile depression following ischemia and reperfusion. J Mol Cell Cardiol 31: 931–947
4. Cifone MG, De Maria R, Roncaioli P, Rippo MR, Azuma M, lanier LL, Santoni A, Testi R (1994) Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J Exp Med 180: 1547–1552
5. Cordia GA, Yoshida T, Das DK (1998) HPTLC analysis of sphingomylein, ceramide, and sphingosine in ischemic/reperfused rat heart. J Pharm Biomed Anal 16: 1189–1193
6. Coroneos E, Martinez M, McKenna S, Kester M (1995) Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines. J Bio Chem 270: 23305–23309
7. Dobrowsky RT, Werner MH, Castellino AM, Chao MV, Hannun YA (1994) Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor. Science 265: 1596–1599

Basic Research in Cardiology, Vol. 96, No. 3 (2001)
© Steinkopff Verlag 2001

8. Frangogiannis NG, Lindsey ML, Michael LH, Youker KA, Bressler RB, Mendoza LH, Spengler RN, Smith CW, Entman ML (1998) Resident cardiac mast cells degranulate and release preformed TNF-alpha, initiating the cytokine cascade in experimental canine myocardial ischemia/reperfusion. Circulation 98: 699–710
9. Gurevitch J, Frolkis I, Yuhas Y, Lifschitz-Mercer B, Berger E, Paz Y, Matsa M, Kramer A, Mohr R (1997) Anti-tumor necrosis factor-alpha improves myocardial recovery after ischemia and reperfusion. J Am Coll Cardiol 30: 1554–1561
10. Gurevitch J, Frolkis I, Yuhas Y, Paz Y, Matsa M, Mohr R, Yakirevich V (1996) Tumor necrosis factor-alpha is released from the isolated heart undergoing ischemia and reperfusion. J Am Coll Cardiol 28: 247–252
11. Haimovitz-Friedman A, Kan CC, Ehleiter D, Persaud RS, McLoughlin M, Fuks Z, Kolesnick RN (1994) Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J Exp Med 180: 525–535
12. Hannun YA (1994) The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269: 3125–3128
13. Hannun YA, Luberto C (2000) Ceramide in the eukaryotic stress response. Trends Cell Biol 10: 73–80
14. Hernandez OM, Discher DJ, Bishopric NH, Webster KA (2000) Rapid activation of neutral sphingomyelinase by hypoxia-reoxygenation of cardiac myocytes. Circ Res 86: 198–204
15. Huwiler A, Pfeilschifter J, van den Bosch H (1999) Nitric oxide donors induce stress signaling via ceramide formation in rat renal mesangial cells. J Biol Chem 274: 7190–7195
16. Jayadev S, Liu B, Bielawska AE, Lee JY, Nazaire F, Pushkareva MY, Obeid LM, Hannun YA (1995) Role for ceramide in cell cycle arrest. J Biol Chem 270: 2047–2052
17. Kim MY, Linardic C, Obeid L, Hannun YA (1991) Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation. J Biol Chem 266: 484–489
18. Kolesnick R, Golde DW (1994) The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. Cell 77: 325–328
19. Li P-L, Zhang DX, Zou A-P, Campbell WB (1999) Effect of ceramide on K_{Ca} channel activity and vascular tone in coronary arteries. Hypertension 33: 1441–1446
20. Li P-L, Zou AP, Campbell WB (1997) Regulation of K_{Ca}-channel activity by cyclic ADP-ribose and ADP-ribose in coronary arterial smooth muscle. Am J Physiol 275: H1002–H1010
21. Liu B, Hannun YA (1997) Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. J Biol Chem 272: 16281–16287
22. Luberto C, Hannun YA (1999) Sphingolipid metabolism in the regulation of bioactive molecules. Lipids 34: S5–S11
23. Mathias S, Younes A, Kan CC, Orlow I, Joseph C, Kolesnick RN (1993) Activation of the sphingomyelin signaling pathway in intact EL4 cells and in a cell-free system by IL-1 beta. Science 259: 519–522
24. Meldrum DR (1998) Tumor necrosis factor in the heart. Am J Physiol 274: R577–R595
25. Meldrum DR, Meng X, Dinarello CA, Ayala A, Cain BS, Shames BD, Ao L, Banerjee A, Harken AH (1998). Human myocardial tissue TNFα expression following acute global ischemia in vivo. J Mol Cell Cardiol 30: 1683–1689
26. Nikolova-Karakashian M, Morgan ET, Alexander C, Liotta DC, Merrill AH (1997) Bimodal regulation of ceramidase by interleukin-1β. Implications for the regulation of cytochrome P450 2C11 (CYP2C11). J Biol Chem 272: 18718–18724
27. Okazaki T, Bielawska A, Bell RM, Hannun YA (1990) Role of ceramide as a lipid mediator of 1 alpha,25-dihydroxyvitamin D3-induced HL-60 cell differentiation. J Biol Chem 265: 15823–15831
28. Oral H, Dorn GW, Mann DL (1997) Sphingosine mediates the immediate negative inotropic effects of tumor necrosis factor-α in the adult mammalian cardiac myocyte. J Biol Chem 272: 4836–4842
29. Priess J, Loomis CR, Bishop WR, Stein R, Niedel JE, Bell RM (1986) Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, hepatocytes, and *ras*- and *sis*-transformed normal rat kidney cells. J Biol Chem 261: 8597–8600
30. Rao BG, Spence MW (1976) Sphingomyelinase activity at pH 7.4 in human brain and a comparison to activity at pH 5.0. J Lipid Res 17: 506–515
31. Schultz JE, Hsu AK, Barbieri JT, Li P-L, Gross GJ (1998) Pertussis toxin abolishes the cardioprotective effect of ischemic preconditioning in intact rat heart. Am J Physiol 275: H495–H500
32. Spence MW, Beed S, Cook HW (1986) Acid and alkaline ceramidases of rat tissues. Biochem Cell Biol 64: 400–404
33. Zager RA, Conrad S, Lochhead K, Sweeney EA, Igarashi Y, Burkhart KM (1998) Altered sphingomyelinase and ceramide expression in the setting of ischemic and nephrotoxic acute renal failure. Kidney Int 53: 573–582