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Effects of ceramide on cardiomyoblasts viability and mitochondrial function

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Von Amir Mohmed Ahmed Abushouk
Geboren am 26.07.1969 in Eldain, Sudan

Betreuer: Junior Prof. Dr. med. S. Rohrbach

Gutachter:

1. Frau Jun.-Prof. Dr. S. Rohrbach
2. Frau Prof. Dr. U. Müller-Werdan
3. Prof. Dr. H. Morawietz (Dresden)

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Abstract and bibliographic description:

Ceramides are sphingolipids that have been shown to regulate several cellular processes including differentiation, growth suppression, cell senescence, and apoptosis. Accumulation of ceramides in aging hearts may play a role in the proapoptotic shifting of Bcl-x splicing and thus accelerates cell death as well as the aging process.

This study was undertaken to investigate the effects of ceramides on the viability of the cardiomyoblast cells (H9C2), the type of cell death that may result from ceramides treatment, whether they affect the balance of the pro and antiapoptotic Bcl-2 family members and mitochondrial function.

H9C2 cells were incubated with or without 25 μM of the synthetic short chain and cell permeable C2 and C6 ceramides, or the long chain C16 ceramide for different time durations. Cell viability, mitochondrial function and some markers of apoptosis were analysed using multiple complementary techniques.

Our results revealed a significant reduction of H9C2 cells viability following incubation with any of the three ceramides for 24 hrs. In comparison to C2 and C16 ceramides, C6 showed effects that are more toxic. All the three ceramides showed reductions in the mitochondrial membrane potential, and an increased cytosolic cytochrome c as a sign of mitochondrial induced apoptosis. Moreover, treatment of the cells with C6 ceramide did result in a significant reduction of mitochondrial complex I, complex I+III, complex III and complex IV activities.

After one hour of ceramides treatment, the cells showed a significant induction of the proapoptotic Bcl-2 family member Bax, this was decreased after 24 hrs. Surprisingly, the cells also showed an increased induction of the antiapoptotic Bcl-xL with an insignificant splicing towards the proapoptotic Bcl-xS. Furthermore, there was increased caspase-9 activity and decreased uncleaved procaspase-3 and 9 following treatment with ceramides, probably as a sign of increased consumption of procaspase-3 and 9. There was no DNA fragmentation observed in response to any of the three ceramides. In conclusion, incubation of H9C2 cells with C2, C6 or C16 ceramides resulted in a programmed cell death, which does not fit with the classical features of apoptosis, and differs depending on the type of ceramide that was applied.

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Content

Abbreviations:

ADP	adenosine diphosphate
AIF	apoptosis inducing factor
AP-1	activating protein-1
Apaf-1	apoptotic protease activating factor-1
APS	Amoniumpersulphate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C2	d-erythro-Sphingosine N-Acetyl
C6	d-erythro-Sphingosine N-Hexanoyl
C8	d-erythro-sphingosine N-Octanoyl
C16	d-erythro-Sphingosine N-Palmitoyl
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CO_2	carbon dioxide
Complex I	NADH-Coenzyme-Q-Oxidoreductase
Complex I+III	NADH- Cytochrome-c- Oxidoreductase
Complex II (SDH)	Succinate Dehydrogenase
Complex II+III	Succinate- Cytochrome-c- Oxidoreductase
Complex III	Ubiquinol-Cytochrome-c- Oxidoreductase
Contr	control
CR	caloric restriction
CS	Citrate synthase
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's culture medium
DMSO	Dimethyl Sulfoxide
DNA	deoxyribonucleic acids
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-(2-amino-ethyl ether) N,N,N´,N´-tetra-acetic acid
FBS	fetal bovine serum
Fig	figure

g	gram
g/l	gram per liter
hr(s)	hour(s)
iNOS	inducible nitric oxide synthase
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-enzimidazolocarbocyanine iodide
kDa	kilo dalton
M	mole
MAPK	mitogen-activated protein kinase
mg	milligram
min	minutes
ml	milliliter
mM	millimole
mRNA	messenger RNA
mt DNA	mitochondrial DNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	Nuclear factor-kappa B
nm	nanometer
NSMase	neutral sphingomyelinases
O_2	oxygen
PBS	Phosphate Buffered Saline
PDGF	platelet-derived growth factor
PI-3-kinase	phosphatidylinositol-3-kinase
PP1	protein phosphatases 1
pmol	picomole
PTP	permeability transition pore
RT	reverse transcription
r.t	room temperature
RNA	ribonucleic acids
ROS	reactive oxygen species
S1P	sphingosine 1-phosphate
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SM	sphingomyelin

SMase	sphingomyelinases
SMCs	smooth muscle cells
SR proteins	serine arginine rich proteins
TBE	Tris-Borate-EDTA buffer
TBS-T	Tris buffered salt solution + tween
TE	Tris-EDTA buffer
TNF	tumor necrosis factor
U	unit
V	volt
v/v	volume per volume
w/v	weight per volume
µl	microliter
µM	micromole
ΔΨm	mitochondrial membrane potential

1. Introduction:

Apoptosis and necrosis are well known causes of the loss of cardiomyocytes in aging heart, a phenomenon that may contribute to the myocardial dysfunction that occurs in the elderly [1].

Recently, data from our laboratory showed a significant shift in hearts of aging rats towards Bcl-xS, one of the proapoptotic isoforms of the Bcl-x gene [2]. This shift towards the proapoptotic Bcl-x isoforms may render cardiomyocytes in culture more susceptible for apoptosis induced by several stimuli and is associated with mitochondrial dysfunction [3].

Interestingly, this proapoptotic alteration in the aging myocardium can be corrected back to the level of the normal value of young animals by a moderate transient caloric restriction (CR), of about 16% reduction of food intake over a period of two months duration [2]. Although it is well known that CR is the most reproducible way to extend lifespan in many species, the basic mechanism of its efficacy remained unclear for a long time [4]. Recently, the new concept of hormesis regards CR as mild stress, which triggers active protective reactions with reparative capacities resulting in expression of genes that can help the cells to cope with a more severe stress [5].

Furthermore, there is increasing evidence that aging is also associated with a failure of liporegulation and accumulation of the sphingolipids like ceramide in senescent cells [6].

Indeed, the observed salutary effects of caloric restriction on life expectancy may therefore reflect a modulation of ceramide contents or ceramide-mediated deleterious effects, which may be increased in the heart and other tissues of older individuals even in the absence of obesity.

1.1. Apoptosis and the aging myocardium:

Apoptosis, or programmed cell death, is a fundamental process that controls normal tissue homeostasis by regulating the balance between cell proliferation and cell death. The role of mitochondria in the regulation of apoptosis, which is triggered by many different stimuli, has been well established and documented [7, 8].

In fact, apoptosis is regulated by multiple factors through complex mechanisms [9]. In general, during the process of apoptosis there are plasma membrane blebbing, cell shrinkage, nuclear condensation, chromosomal DNA fragmentation and reduction in the mitochondrial transmembrane potential (ΔΨm). This is followed by the release of many apoptotic inducers such as apoptosis inducing factor (AIF) and cytochrome c from the mitochondrial intermembrane space into the cytosol of cells undergoing apoptosis [10-14]. Cytosolic cytochrome c then forms a complex with apoptotic protease activating factor-1 **(**Apaf-1) and procaspase-9, resulting in activation of caspase-9, which processes and activates other caspases, such as caspase-3, leading to the execution of programmed cell death [15]. It is well known that many inducers of apoptosis activate different caspases [16].

Moreover, Bcl-2 family proteins also serve as critical regulators of mitochondrial apoptosis by functioning as either inhibitors or promoters of cell death [17, 18]. Proapoptotic Bcl-2 family proteins, including Bcl-xS, Bax, Bak and Bid, induce mitochondrial membrane permeabilization and cytochrome c release [19-21]. In contrast, the antiapoptotic Bcl-2 family proteins, like Bcl-xL and Bcl2, are capable of preventing cytochrome c release and can also significantly inhibit cell death, which is mediated by the proapoptotics Bax and Bid, through prevention of channel formation [22-25]. It was found that in healthy cells Bcl-2 adopts a typical tail-anchored topology. Inducers of apoptosis like ceramide and etoposide trigger change of Bcl-2 to the multispanning transmembrane topology [26]. In addition to membrane topology, phosphorylation of Bcl-2 is required for its full antiapoptotic function [27, 28]. However, the Bcl-2 proteins are becoming increasingly recognized as important modulators of cardiomyocytes apoptosis and their mRNA is expressed in both developing and adult hearts [29-32]. Regulation of these proteins was found to be important for apoptosis induced by oxidative stress in cultured cardiomyocytes [33].

Several factors that regulate apoptosis have splice variants with an opposite negative function. Previous studies have demonstrated that several splice variants are derived from both caspase-9 and Bcl-x genes in which the Bcl-x splice variant Bcl-xL and the caspase-9 splice variant caspase-9b inhibit apoptosis, in contrast to the proapoptotic splice variants Bcl-xS and caspase-9 [34, 35].

Our group has shown a significant enhancement of the Bcl-xS/ Bcl-xL protein ratio in ventricular myocardium of aging rats [36], providing an evidence to explain the

age-associated mitochondrial dysfunction in the aging myocardium. Similarly, in failing human myocardium an elevated Bcl-xS/ Bcl-xL protein ratio was observed [3]. It is now clear that the balance between the pro and antiapoptotic Bcl-2 proteins products plays a crucial role in the cardiomyocytes mitochondrial function, either rendering them into apoptotic shift or provoking their survival.

In cardiomyocytes apoptosis has been demonstrated after injury caused by ischemia and reperfusion [37, 38], myocardial infarction [39, 40], ventricular pacing [41], coronary embolization, heart failure, and cardiac aging [1, 42, 43].

The theory of mitochondrial aging could be implicated in apoptosis of aging heart. This theory links production of reactive oxygen species (ROS), mtDNA damage and respiratory chain dysfunction in a vicious cycle that generates a progressive decline of mitochondrial function in aging cells. This eventually impairs cellular function and viability and the cycle seems to be subjected to modulation and acceleration by many influencing factors, among them are the Bcl-2 family proteins [5].

During the last years ceramide attracted attention as a potential inducer of apoptosis. Therefore, a large body of research focused on the role of ceramides on the different cellular functions.

1.2. Ceramides:

Ceramides are sphingolipids that have been described as messengers for several events like differentiation, senescence, proliferation and cell cycle arrest in different cell lines [44, 45].

1.2.1. Synthesis and metabolism of ceramide:

Membrane lipids of the sphingolipid class contain a long-chain sphingoid base backbone (such as sphingosine), linked to a fatty acid chain via an amide bond, and one of various polar head groups. The structure of these head groups defines the various sphingolipid subtypes; with a hydroxyl group in ceramide, phosphorylcholine in sphingomyelin (SM), and carbohydrates in glycosphingolipids.

Sphingolipids are found in most subcellular membranes. In the plasma membrane they are predominantly found in the outer leaflet [46, 47]. The metabolism of sphingolipids has been proved to be a dynamic process and their metabolites (such as ceramide, sphingosine, and sphingosine 1-phosphate (S1P)) are now recognized as messengers playing essential roles in cell growth, survival, as well as cell death [45, 48, 49].

Sphingomyelin (SM) is a ubiquitous component of animal cell membranes, where it is by far the most abundant sphingolipid. Indeed, it may comprise as much as 50% of the lipids in certain tissues and it is particularly abundant in the nervous systems of mammals [50].

Ceramide can be formed through sphingomyelinases (SMase)-dependent catabolism of SM and by de novo synthesis (Fig. 1). SMases are specialized enzymes with phospholipase C activity that can hydrolyze the phosphodiester bond of SM. Several isoforms of SMases can be distinguished by their different pH optima. Acid SMase is a lysosomal enzyme, its deficiency was shown to be associated with degenerative changes in the nervous system and resistance to radiotherapy [2, 51]. Neutral SMase (NSMase) is associated with the plasma membrane and can be activated in response to a variety of stimuli, promoting an increase in cellular ceramide levels over a period of minutes to hours [44, 45]. Alkaline SMase activity is found in the intestinal mucosa and bile, it does not appear to participate in signal transduction [52, 53].

De novo ceramide biosynthesis requires coordinated action of serine palmitoyl transferase and ceramide synthase to generate ceramide (Fig. 1). This process begins with the condensation of serine and palmitoyl-CoA, catalysed by the enzyme serine palmitoyl transferase to form 3-ketosphinganine [45, 48, 54]. This is then reduced to the sphingoid base sphinganine and acylated by ceramide synthase to generate dihydroceramide. Introduction of the 4,5-*trans*-double bond of sphingosine into dihydroceramide leads to the generation of ceramide catalyzed by the enzyme dihydroceramide desaturase [55]. Alternately, this pathway may re-utilize the sphingosine released by a sequential degradation of more complex sphingolipids for ceramide synthesis.

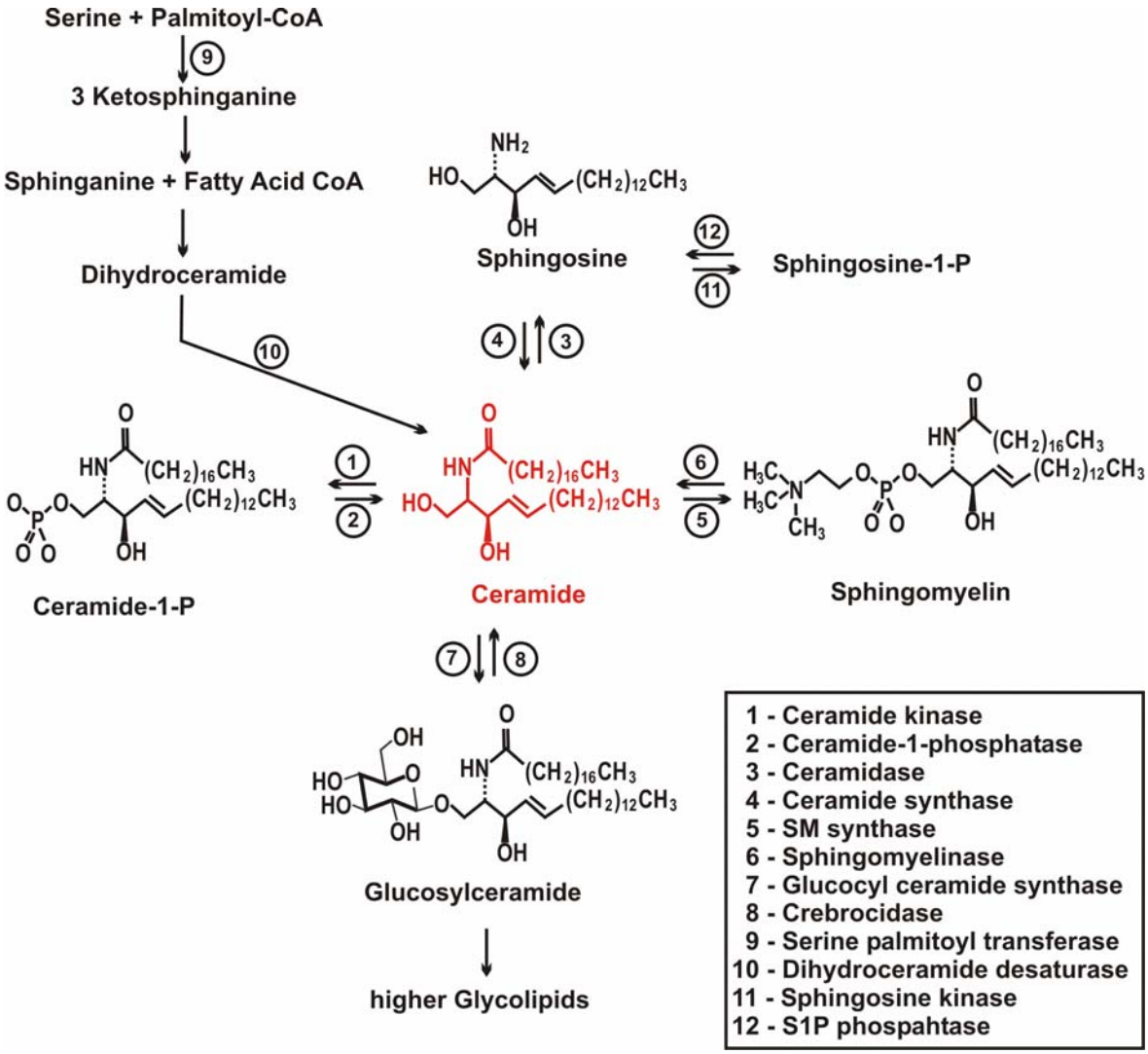

Figure 1: Ceramide metabolic pathways

Ceramide can be converted into a variety of metabolites; phosphorylation by ceramide kinase generates ceramide-1-phosphate, while deacylation by either neutral or acid ceramidase yields sphingosine, which can be phosphorylated by sphingosine kinase to S1P [45, 48].

Ceramide can also be converted back to SM by the transfer of phosphorylcholine from phosphatidylcholine to ceramide via SM synthase [48]. Alternatively, it can be glycosylated by glucosylceramide synthase to form glucosylceramide, which may be further modified by various enzymes in the Golgi apparatus to form complex glycosphingolipids.

It is reported that sphingomyelin, which is the major phospholipid located in the cellular membranes of myocytes, can be broken down during ischemia and reperfusion, generating intracellular ceramide and sphingosine in the heart through acidic or neutral sphingomyelinase [56, 57]. Ceramide can also be produced in the

heart through a de novo synthesis pathway catalyzed by ceramide synthase [45]. Therefore, ceramide accumulation was found to cause different cardiovascular effects, including the cardiac dysfunction in obesity, increasing the incidence of thrombosis by affecting tissue factor and plasminogen activator inhibitor release [58-60]. Furthermore, ceramide was implicated in the cardiac induced apoptosis and microvascular damage in response to anticancer therapy [61, 62].

Inducers of ceramide accumulation include a number of stressors, such as various cytokines, serum deprivation, and dietary sphingolipids that could be hydrolyzed throughout the small and large intestine to bioactive compounds, such as sphingosine and ceramides [63-66]. Moreover, up-regulation of p53 in response to chemotherapeutic agents or y-irradiation can also induce the accumulation of cellular ceramides [51, 67, 68]. These observations led to the suggestion that ceramide may play a role as a coordinator of cellular responses to stress [69, 70].

1.2.2. Cellular targets for ceramides:

It is well known that ceramide can modulate many different cellular processes; in this context we will focus on some of the pathways through which it affects the cell death and survival. A number of protein targets for ceramide that regulate cell death process have been identified, with ceramide-activated protein kinases and phosphatases being the most extensively studied [71, 72]. Ceramide directly regulates protein phosphatase 1 (PP1), inducing dephosphorylation of SR proteins and splicing of caspase-9 and Bcl-x genes [34].

Interaction of ceramide with protein kinase-c can inhibit translocation of the kinase to the plasma membrane and therefore inhibits its catalytic activity. In response to ceramides, activation of an atypical isoform of protein kinase-c can mediate activation of NF-κB, which is sensitive to various types of cellular stresses including oxidative and metabolic stresses [73-75].

It was also observed that ceramide specifically binds to and activates protein kinase c-Raf, leading to a subsequent activation of the classical MAPK cascade [76].

Treatment with ceramide was also reported to cause an increased activity of c-Jun-N-terminal protein kinase (JNK; also known as stress-activated protein kinase) and increase DNA binding of activating protein-1 (AP-1), a nuclear transcription

factor, which was shown to have multiple functions in cellular regulation including proliferation, differentiation and apoptosis [77-79].
Ceramide was also found to interact directly with phospholipase A2 facilitating its inflammatory actions [80].
Cathepsin D also links ceramide to the induction of apoptosis, where binding of ceramide to cathepsin D in endosomes or in vitro triggers autocatalytic cleavage of cathepsin to its active form. A link between cathepsin D and apoptosis was originally defined by Kimchi and co-workers, who showed that cells transfected with antisense cathepsin D mRNA, did survive exposure to apoptotic stimuli [80-82].
Such intimate links between stress, ceramide production, and the different cellular stress responses are consistent with a role for SM-ceramide signalling in cell death and survival.

1.2.3. Ceramides and cell death:

It is now well established that in response to many cytotoxic and chemotherapeutic agents ceramide is an endogenous regulator of apoptosis in cardiomyocytes. Treatment of cardiomyocytes in culture with synthetic ceramides was able to induce apoptosis and inhibit cellular proliferation. Also apoptosis induced by ischemia-reperfusion or treatment with tumor necrosis factor-α in cardiomyocytes was preceded by a significant elevation in ceramide levels [56, 83, 84].
It has been shown that ceramide can alter mitochondrial function by two major pathways, either indirectly or directly. Indirectly, ceramide modifies the activity of proapoptotic and antiapoptotic members of the Bcl-2 family proteins, which in turn alter the outer mitochondrial membrane permeability for cytochrome c and other proapoptotic molecules. In this pathway targets of ceramide are non-mitochondrial molecules like cathepsin D, which triggers translocation of Bax to the mitochondria [85, 86], and serine-threonine protein phosphatase 2A, which dephosphorylates Bcl-2, thereby decreasing its antiapoptotic activity [72]. An additional substrate for protein phosphatase 2A is the serine-threonine Akt-protein kinase B [85]. Protein phosphatase 2A inactivates Akt, resulting in dephosphorylation and activation of the proapoptotic Bad [87]. It was also found that endogenous ceramide and PP1 regulate alternative splicing of Bcl-x and caspase-9. When the cell-permeable C6

ceramide was added to the lung cancer cells A549 in culture, it resulted in a down-regulation of the antiapoptotic splice variant Bcl-xL and caspase-9b with concomitant increased levels of Bcl-xS and caspase-9 in a dose- and time-dependent manner [34]. Thus, the overall effect of ceramide on this pathway is an increase in the proapoptotic proteins bound to mitochondria.

Evidence is accumulating to implicate direct actions of ceramide on the mitochondria in intact cells. Birbes and co-workers found that the selective hydrolysis of the mitochondrial pool of sphingomyelin by bacterial sphingomyelinase, targeted to the mitochondrial matrix, resulted in apoptosis. Whereas production of ceramide in the plasma membrane, endoplasmic reticulum, nucleus and Golgi apparatus by bacterial sphingomyelinase targeted to these compartments had no effect on cell viability [88].

Moreover, several researches did focus on the ability of ceramides to release cytochrome c from the mitochondrial intermembrane space and to induce permeability of the inner mitochondrial membrane, permitting passage of low molecular solutes. In the models of Siskind and co-workers, the outer mitochondrial membrane was considered as a primary target for ceramides, which induce cytochrome c release, whereas the inner membrane was viewed as being ceramide-insensitive [89-91]. In contrast, Pastorino and Szalai suggested that the opening of PTP in the inner mitochondrial membrane could be a primary event in the initiation of cytochrome c release and in increasing solute permeability of the inner membrane in the presence of ceramides [92, 93]. In the same context, Di Paola and co-workers provided evidence for the role of ceramide as a non-specific modulator of ionic permeability of the lipid component of the inner mitochondrial membrane [94]. Thus, the proposed mechanisms by which ceramides alter mitochondrial membrane permeability are varied, and the localization of functionally significant ceramide targets (outer mitochondrial membrane, inner membrane, or matrix space) is unclear.

Recently, Novgorodov and co-workers developed derivatives of ceramide with a fixed positive charge. A strategy by which ceramide can be selectively delivered to the mitochondrial matrix, based on their electrochemical potential, to probe its mechanisms of action [95]. They showed that these positively charged ceramides did increase the permeability of the inner membrane (decrease in $\Delta\Psi m$), which in turn resulted in the release of cytochrome c. In addition, they found that ceramide-

induced permeabilization of the inner mitochondrial membrane was mediated by specific ion transport systems, namely the PTP and electrogenic H^+ transporter.
Di Paola and co-workers did a study on the interaction of ceramide with the respiratory chain of mitochondria isolated from rat heart, comparing the effects elicited by short and long-chain ceramides C2 and C16, respectively. They found that both ceramides inhibited the activity of complex I and the oxidation of NADH-linked substrates in addition to the inhibition of mitochondrial succinate oxidase activity. Furthermore, also they found that short-chain ceramide caused collapse of the mitochondrial membrane potential, whereas long-chain ceramide did not. It has also been reported in another study, which was performed on isolated rat heart mitochondria, that C2 ceramide could directly reduce the activity of respiratory chain complex III [94, 96, 97].
Although ceramides are well known to cause cell death by inducing apoptosis, they can also cause non-apoptotic cell death depending on the model system and the experimental conditions. However, ceramides were found to cause cell death by necrosis in different cancer cell lines [98-100].

1.2.4. Ceramides and promotion of cell survival:

Ceramide emerges as a pleiotropic biologic activator capable of inducing two mutually exclusive cellular functions, cell proliferation and cell death.
Exogenous C2 and C6 ceramides were able to induce DNA synthesis and proliferation of quiescent Swiss 3T3 fibroblasts. Similar effects were observed with addition of natural bovine brain ceramides [101, 102].
Interestingly, low concentrations of C2 ceramide did promote survival of cultured embryonic rat brain neurons, whereas higher concentrations induced apoptosis [103].
Another example for a positive proliferative effect is stimulation of growth of bovine aortic smooth muscle cells following treatment with ceramide in culture, through activation of MAPK pathway [104].
Moreover, ceramide was found to be able to enhance cell proliferation through its metabolites such as Sphingosine-1-p (S1P), which is known to transmit survival signals by activating PI-3-kinase, Akt and Bcl-2 [105].

Intracellularly produced ceramide and S1P stimulated DNA synthesis in endothelial and smooth muscle cells (SMCs). They also potentiated the mitogenesis induced by various growth factors including platelet-derived growth factor (PDGF) [106].

Furthermore, ceramide could exhibit a protective role against ischemic injury in some cases of ischemia reperfusion, as reported in heart or liver [107-109].

The fact that ceramide and other SM metabolites can influence the proliferation, differentiation, survival of many different types of cells, and their involvement in stress signalling pathways, strongly suggests a fundamental role for SM and its bioactive products in aging. The splicing of the Bcl-x gene associated with shifting toward apoptosis in aging rats hearts and its renormalization by caloric restriction, led to the assumption that this might result from ceramide accumulation. Approval of this hypothesis raises two important questions, first, is there a significant accumulation of ceramide in aging hearts, and what is the effect of caloric restriction on that? (Another group from our lab will answer this question). The second question is, does accumulation of ceramide in a cell culture model lead to apoptosis associated with Bcl-x gene splicing in cardiomyocytes?. The answer to this question will be the target of this thesis, which will discuss the effects of addition of exogenous cell permeable ceramides on cardiac cells in culture, and to find out to what extend ceramides can modulate Bcl-2 family members and cell survival.

2. Objectives of the study:

The objectives of this study were to investigate the effects of exogenous ceramides on the rat ventricular cardiomyoblast cell line H9C2, especially on the:

1) cell viability.
2) type of cell death triggered by ceramides.
3) the induced phenotype changes in the balance of pro and antiapoptotic determinants.
4) mitochondrial function.

In spite of the fact that the rat ventricular cardiomyoblast cells are not typical adult cardiomyocytes, they were used in this study as a cellular model to investigate the effects of accumulation of ceramide on the heart. H9C2 cells represent a stable cell line, with characteristics very similar to that of the skeletal muscle myoblast. In contrast, preparation of the neonatal cardiomyocytes represents an inhomogeneous cell culture, while adult cardiomyocytes dedifferentiate and undergo apoptosis in culture. In this study, exogenous cell permeable short chain ceramides like C2 and C6 were used as well as the long chain ceramide C16.

3. Materials and methods:

3.1. Cell culture:

The rat myoblastic ventricular myocardial cells H9C2 were purchased from the American type culture collection (ATCC). They were cultured in Dulbecco's modified Eagle's medium (DMEM), which contains 4.5 g/l glucose and 3.7 g/l Na_2CO_3. The medium was supplemented with 100 U/ml Penicillin and Streptomycin, 10% fetal bovine serum (FBS), 1 mM Na-pyruvate and 4 mM glutamate (Biochrom AG, Berlin, Germany).
The cells were grown in a humidified incubator with 5% CO_2 and at 37°C. They were passaged regularly and subcultured to 80-90% confluence before any experimental procedures.
For isolation of DNA, RNA and proteins, $1x10^6$ H9C2 cells were seeded in 10 cm² dishes filled with 10 ml DMEM. After overnight incubation, the cells were treated with ceramides; d-erythro-Sphingosine N-Acetyl (C2), d-erythro-Sphingosine N-Hexanoyl (C6) or d-erythro-Sphingosine N-Palmitoyl (C16) at a concentration of 25 µM. The treatment duration for DNA was 24 hrs, RNA 1, 6, 16 or 24 hrs, and for proteins (total and cytosolic fraction) was 24 hrs. The cells were scraped, together with detached cells, and were collected by centrifugation at 1400 xg for 5 minutes, and then the pellets were washed with warm PBS.

3.2. Cell viability assay:

The toxic effects of ceramides on H9C2 cells were tested by using MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) conversion assay, which is based on reduction of the yellow MTT to purple formazon by living cells.
In 96 well plates $1x10^4$ H9C2 cells were seeded in 100 µl DMEM per well, the outer wells were excluded from seeding and filled with medium only. One day after seeding, the cells were treated with serial dilutions of C2, C6 or C16 ceramides, from 50 µM to 1.6 µM for 24 hrs, and 16 wells were left as untreated controls. After 24 hrs of treatment with ceramides, the medium was changed and fresh medium containing 1 µg/ml MTT (100 ml fresh culture medium and 25 µl of 5 mg/ml MTT in PBS) was added. Two hrs later 100 µl lysis buffer (10 g SDS/100 ml, 99.4 ml

DMSO, 0.6 ml acetic acid) were added to each well and incubated for one hr at 37°C. Then the absorbance of the solubilized MTT formazan products was measured in a microplate reader photometer (Dynatech, Germany) at 570 nm. For all the concentrations used from the different ceramides the mean of six readings was calculated, non-cellular background was subtracted and the results were calculated to the MTT conversion of untreated control cells, which corresponds to 100% survival.

3.3. DNA isolation:

For isolation of the DNA, Purogene® DNA purification kit (Gentra, Minneapolis, USA) was used, and the manufacturer's protocol for 1-2 million cultured cells was followed. The DNA pellets were hydrated with 50 µl hydration solution, and then the DNA concentration was photometrically measured before any further manipulation or storage at 4°C.

For separation of the DNA by electrophoresis, the samples were mixed with sample buffer (50% (v/v) 1X Tris-EDTA (TE) buffer [10 mM Tris-HCL pH 8.8; 1 mM EDTA, pH 8.0]; 50% (v/v) glycerol; 0.25% (w/v) bromophenol blue). Then loaded in 1% (w/v) agarose gel (1% (w/v) agarose heated in 1X TBE buffer [89 mM Tris – HCl, pH 8.0; 89 mM Boric acid; 2.5 mM EDTA, pH 8.8]) and containing 0.5 µl/ml of 10.000 X Sybr-green. The gels were run at 80-120 V, and the sizes of the bands were determined using a 100 bp DNA ladder (Invitrogen, Karlsruhe, Germany).

3.4. RNA isolation:

The cells pellets were dissolved in 300 µl TRI-Reagent® (Sigma, Taufkirchen, Germany) and the manufacturer's protocol was followed. After isolation, the RNA pellets were dried and dissolved in 30 µl sterile DEPC water. The RNA was spectrophotometrically measured. The quality of the RNA was checked with 1% (w/v) agarose gel electrophoresis. After isolation and measurement, the RNA was stored at -80°C for long-term storage.

3.5. Photometric quantification of DNA and RNA:

The quantities of DNA and RNA were photometrically analysed with their absorbance at 260 nm and 280 nm (Ultrospec® 2000, Pharmacia Biotech. Upsala, Sweden).

3.6. Reverse transcription (RT):

500 ng of RNA were diluted in 10 µl DEPC H_2O and denatured at 72°C for 3 min. Then 15 µl of a master mix (Promega, Madison, WI, U.S.A) with the contents shown in table (1) were added to the samples and incubated for 30 min at 42°C, then the temperature was raised to 95°C for one min, followed by cooling to 4°C.

Table 1: RT master mix content.

Substance	Volume in µl
30 µg/ml random primers	3.0
M-MLV RT 5 X buffer	5.0
12.5 mM dNTP	1.0
40 U/µl RNase out (Ribonuclease inhibitor)	0.25
200 U/µl Reverse transcriptase	0.25
DEPC dH_2O	5.5

3.7. Polymerase chain reaction (PCR):

The complementary DNA (cDNA), which was synthesized by reverse transcription, was further used in semiquantitative PCR to detect Bcl-xL/S, Bax and 18S rRNA.
All PCRs were done in T3 and T-Gradient thermocyclers (Biometra-Germany), in 600 µl PCR tubes containing 25 µl of a mixture as shown in table (2). The primers that were used are shown in table (3). The annealing temperature and the number of cycles were optimized as shown in table (4).

Table 2: PCR master mix.

Substance	Volume in µl
10X Pfu buffer	2.5
100 µM dNTP mix	3.0
10 pmol/µl sense primer	0.5
10 pmol/µl anti sense primer	0.5
5 U/µl Pfu polymerase	0.25
cDNA	2.0
dH_2O	16.25

Table 3: The primers sequences.

Gene	Sequence of the sense primer	Sequence of the anti sense primer
Bax	ATGTTTGCAGATGGCAGCTT	TCAGCCCATCTTCTTCCAGA
Bcl-xL/S	CTGAGAGAGGCTGGCGATC	GTCAGGAACCAGCGGTTGA
18S rRNA	GTTGGTGGAGCGATTTGTCT GG	AGGGCAGGGACTTAATCAACGC

Table 4: Annealing temperatures.

Primer	Annealing temperature in °C	Number of cycles
Bax	60	40
Bcl-xL/S	60	30
18S rRNA	60	10

After amplification, 25 µL of each PCR product, along with a ladder of known molecular weights (100 bp DNA ladder (Invitrogen, Germany)), were electrophoresed using 1X tris-boric-ethylene diamine tetraacetic acid (TBE) buffer in 1% agarose gel (1% (w/v) agarose heated in 1X TBE buffer [89 mM Tris–HCl, pH 8.0; 89 mM Boric acid; 2.5 mM EDTA, pH 8.8]) containing 1 µg/mL ethidium bromide. Before loading the gels, the samples were mixed with sample buffer

(50% (v/v) 1X Tris-EDTA (TE) buffer [10 mM Tris-HCL, pH 8.8; 1 mM EDTA, pH 8.0]; 50 % (v/v) glycerol; 0.25 % (w/v) bromophenol blue). The resulting bands were photographed and analysed with Biostep gel documentation system.

3.8. Proteins extraction and measurement:

3.8.1. Extraction of total cell proteins:

For the extraction of total proteins, the cells pellets were dissolved in 200 µl SDS lysis buffer (1 M Tris-HCL, pH 7.5; 10% (v/v) Sodiumdodecylsulfate (SDS), 4 M NaCL and freshly added 1:100 protease-inhibitor-mix). Then all following procedures were performed on ice. The cells were sonicated for 10 seconds then centrifuged for 20 minutes at 14000 rpm at 4°C, the supernatants were taken into new tubes and the protein concentration was measured.

3.8.2. Extraction of the cytosolic protein fraction:

For extraction of the cytosolic protein fraction, the cells pellets were resuspended in 100 µl of sucrose buffer (10 mM Hepes, pH 7.4; 250 mM sucrose, 80 mM KCL, 1 M EDTA, 1 M EGTA, 4 M fresh DTT, saponine and fresh 1:200 protease inhibitor). After incubation on ice for 30 minutes and centrifugation for 10 min at 500 xg and 4°C, supernatants were transferred to new tubes and centrifuged for 30 min at 10000 xg and 4°C. Then the supernatants that contained the cytosolic protein fraction were taken into a new set of tubes.

Protein concentrations were estimated with a micro plate-reader photometer (Dynatech, Germany) at a wave length of 570 nm, using the BCA™ Protein assay kit and bovine serum albumin (BSA) as standard (Pierce, Rockfort, USA) according to the manufacturer's manual.

Each 50 µg or 100 µg proteins were added to 6 x SDS buffer (0.66 g DTT; 0.4 g SDS; 6 ml glycerol; 2 ml 1.25 M Tris-HCl, pH 6.8; 0.2 mg bromphenolblue; and 10 ml dH_2O) and stored at -20°C for further manipulation or at -80°C for long term storage.

3.9. SDS-polyacrylamide gels:

For immunoblot analysis, 50 µg of cellular proteins were electrophoresed through 10% SDS-polyacrylamide gels. The gels were prepared using mini-protean II cell system (Bio-Rad, Munich, Germany) as shown in table (5).
The protein samples were loaded in the gel after being denatured at 95°C for 5 min in the 1x loading buffer (6 x SDS: 0.66 g DTT; 0.4 g SDS; 6 ml glycerol; 2 ml 1.25 M Tris-HCl, pH 6.8; 0.2 mg bromphenolblue; and 10 ml dH_2O). At the same time protein marker was loaded for estimation of the molecular size of the bands.
The gels were run at a voltage of 14 V/cm and as soon as the bromophenol blue colour enters the running gel, the voltage was increased to 24 V/cm.

Table 5: Polyacrylamide gels preparation.

Substance	5% stacking gel	10% running gel
40% (w/w) acrylamide.	0.5 ml	3.0 ml
Stacking gel buffer (1 M Tris –HCL, pH 6.8).	0.5 ml	
Running gel buffer (1 M Tris –HCL, pH 8.8).		4.5 ml
dH_2O.	2.95 ml	4.3 ml
10% (w/v) SDS.	40 µl	120 µl
10% (w/v) Amoniumpersulphate (APS).	25 µl	100 µl
TEMED.	5 µl	10 µl

3.10. Western blots:

The proteins were transferred onto nitrocellulose membranes (0.45 µM, Protran® Schleicher and Schuell Bio Science GmbH Whatman group, Dassel, Germany), using the mini blot transfer cell-system (Bio-Rad, Munich, Germany), according to the protocol of the company.
After blotting for 1 hr at 100 V in a transfer buffer (25 mM Tris-HCL, pH 8.2; 192 mM glycin, 20% (v/v) methanol, 0.1% (v/v) SDS), the membranes were stained with Ponceau S solution to confirm equal protein loading (0.5 g Ponceau S

dissolved in 1 ml acetic acid and the volume was completed to 100 ml with dH_2O). The equal transfer was checked with staining of the gels with coomassie staining solution (0.5 g coomassie brilliant blue G-250 dissolved in 500 ml methanol, and then 100 ml acetic acid and 400 ml dH_2O were added).

The membranes were washed with dH_2O from the Ponceau S and blocked with 5% skim milk dissolved in TBS-T buffer (10 mM Tris-HCL, pH 7.3; 500 mM NaCL, 0.2% (v/v) tween 20), and shaken for 2 hrs at room temperature (r.t). Then the membranes were washed three times (5 min each time) with TBS-T buffer.

After that, the membranes were incubated overnight at 4°C with one of the primary antibodies shown in table 6, which were diluted in a solution containing TBS-T buffer, 2.5% (w/v) skim milk and 0.02 % (w/v) NaH_3.

After that the membranes were washed three times (10 min each time) with TBS-T buffer at r.t, then incubated for one hr at r.t with the appropriate secondary antibodies, which were diluted in 2.5 % skim milk in TBS-T (Table 7).

Table 6: Primary antibodies.

Antibody	Source	Manufacturer	Dilution
Bax	Rabbit polyclonal	Cell Signaling	1:750
Bcl-xL/S	Rabbit polyclonal	Santa Cruz Biotechnology	1:500
Caspase-3	Rabbit monoclonal	Cell Signaling	1:750
Caspase-9	Rabbit polyclonal	Cell Signaling	1:500
Cytochrome c	Mouse monoclonal	BD Pharmingen	1:1.000
GAPDH	polyclonal	abcam	1:5.000

Table 7: Secondary antibodies.

Antibody	Source	Manufacturer	Dilution
Anti rabbit	Goat monoclonal, horseradish peroxdase labelled	Santa Cruz Biotechnology	1:10.000
Anti mouse	Goat monoclonal, horseradish peroxdase labelled	Santa Cruz Biotechnology	1:10.000

Chemiluminescence detection was performed by incubating the membranes for 1 min in a freshly prepared mixture from reagent 1 and reagent 2 of the ECL western blotting detection reagents (Amersham Bioscience, UK), followed by exposure to X-ray films (Hyper Films TM, Amersham Bioscience, UK).

3.11. JC-1 analysis for determination of the mitochondrial membrane potential:

H9C2 cells were seeded at 6 x 10^4 cells/well in a 6-well plate. The following day the cells were incubated for 24 hrs with 25 μM C2, C6 or C16 ceramides and a control group in fresh medium without treatment. At the end of the incubation, the cells were rinsed twice with prewarmed PBS and incubated with 10 μg/ml JC-1 fluorochrome (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolocarbocyanine-iodide, (Molecular Probes , USA)) in DMEM for 10 min at 37°C. JC-1 stock solution, at a concentration of 5 mg/ml, was prepared in DMSO and kept in darkness at -20°C. Again, the cells were rinsed twice with prewarmed PBS, then they were examined using Axiovert 200 M microscope (Carl Zeiss GmbH, Jena, Germany), assisted with visitron software system (Visitron system GmbH, Puchheim, Germany). For visualization of the green fluorescence of JC-1 monomers, a 450-490 nm excitation wave length was used. The red fluorescence of JC-1 aggregates was visualized using a 590 nm wave length. For a positive control assay, valinomycin was used in a concentration of five μM and 10 min incubation to allow complete dissipation of the membrane potential.

3.12. Caspase-9 activity assay:

H9C2 cells were seeded in opaque 96-well plates (2 x 10^4 cells/well) and treated with C2, C6 or C16 ceramides for 6,16, or 24 hrs time intervals, control cells were left untreated. According to the manufacturer's protocol, 50 µl of Caspase-Glo® 9 reagent (Promega) were added to each well (1:1 ratio of Caspase-Glo® 9 Reagent volume to sample volume). In addition, 50 µl of the reagent were added to blank wells containing only medium. The blank reaction was used to measure background luminescence associated with the culture medium and the Caspase-Glo® 9 reagent. The luminescence value for the blank reaction was subtracted from experimental values.
The luminescence of each sample was measured in a plate-reading luminometer (GLOMAX-Turner Bio System) as directed by the luminometer manufacturer.
Increase in caspase-9 activity was calculated based on the activity measured from untreated cells, after subtraction of the blank values.

3.13. Determination of mitochondrial enzyme activity:

After 24 hrs of incubation of H9C2 cells with 25 µM C6 ceramide, the cells were washed with PBS, trypsinized and centrifuged at r.t for 5 min at 2000 rpm. Then a homogenate of 20x10^6 /ml of Chappel-Perry buffer [50 mM of Tris buffer (pH 7.5), 100 mM potassium chloride, 5 mM $MgCl_2$, and 1 mM ethylenediaminetetraacetic acid (EGTA)] was performed using a glass/glass homogenizer (Kontes Glass Co., Vineland, New Jersey, 2 ml, 0.025 mm clearance).
Enzyme activities were assayed at 30°C spectrophotometrically using a DU 640 photometer (Beckmann Instruments, Palo Alto, California). Each assay was performed at least as duplicate with two different volumes of cells homogenate, related to citrate synthase activity.
The analysis of the mitochondrial respiratory chain complexes; NADH-ubiquinone reductase (complex I), NADH-cytochrome c reductase (complex I+III), succinate-cytochrome c reductase (complex II +III), ubiquinol-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) were measured following the reduction of cytochrome c at 550 nm (after the addition of NADH or succinate). Using an extinction coefficient for cytochrome c of 19.2 µM/cm. Succinate

dehydrogenase (complex II) was measured by recording the reduction of 2,6-dichlorophenolindophenol at 600 nm using an extinction coefficient of 19.1 µM/cm. Citrate synthase was determined with Nbs_2, oxaloacetate, acetyl-CoA and 0.1% Triton X-100 at 412 nm using a problem extinction coefficient of 13.6 µM/cm [110-112].

3.14. Statistical analysis:

The results were shown as mean values ± SEM. To test for statistical differences, the t-test and one-way ANOVA were used with the help of the Sigma Stat software V 3.1 (Sigma Stat Software Inc., Chicago, USA.). P values < 0.05 were considered significant.

4. Results:

4.1. Influence of ceramides on cell viability:

Cell survival was assessed by a MTT conversion assay and normalized to the MTT activity of the untreated control cells which were defined as 100% survival.
Incubation of H9C2 cells in culture for 24 hrs with the exogenous ceramides C2, C6 or C16 resulted in the reduction of cell viability in a time and concentration dependent manner (Fig. 2).
The cells showed comparable responses to the three ceramides at lower concentrations. However, when the concentration of ceramides higher than 25 µM, the viability dropped rapidly especially in response to C6.
The viability of H9C2 cells after 24 hrs treatment with 50 µM concentration of C2, C6 or C16 ceramides was 46%, 9% and 47% of the untreated control cells, respectively. Indicating that more toxic effects of C6 ceramide on this cell line at this concentration and time.
After four independent experiments we continued to use a 25 µM concentration of the ceramides in the subsequent experiments, where the cell viability was found to be 71%, 63% and 78% of the untreated control cells for C2, C6 and C16, respectively.

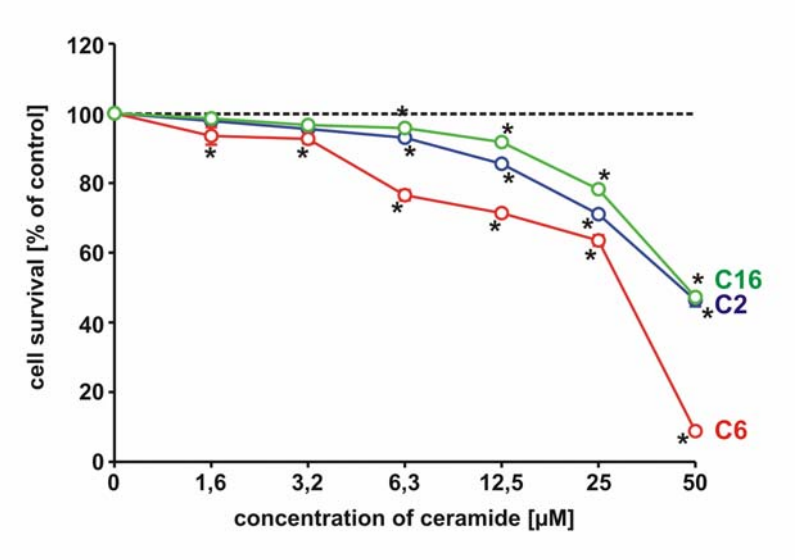

Fig. 2: Dose dependent effects of ceramides on cell survival: H9C2 cells were exposed to various concentrations of C2, C6 or C16 ceramides for 24 hrs. Cell survival was estimated by MTT assay, as described in the materials and methods section. Control cells were defined as 100% survival. The data are mean values ± SEM of 4 independent experiments performed in duplicate, (* P< 0.05).

4.2. Effects of ceramides on the expression of the Bcl-2 family:

The Bcl-2 family consists of proapoptotic proteins such as Bax, and Bcl-xS, and death-suppressors such as Bcl-xL.

4.2.1. Effects of ceramides on Bax:

To study the effects of ceramides on the expression of mRNA of the proapoptotic Bax, H9C2 cells were incubated with or without 25 μM C2, C6 or C16 ceramides for 1, 6, 16 or 24 hrs (Fig. 3 a). After one hour of incubation, the cells showed an induction of Bax in response to the three ceramides, which was found to be about two and four times higher in C2 and C16 than in the control cells, respectively. This induction of Bax reached a peak after six hrs in case of C6 and C16, decreased after 16 hrs to reach the lowest levels after 24 hrs, even below the values of the untreated cells. The response of the cells to C2 was different after 6 hrs of treatment. They showed a decline in the induction of Bax mRNA, which continued to decrease similar to C6 and C16 to be below the level of the untreated cells after 24 hrs.

On the other hand, the immunoblots obtained from the incubation of H9C2 cells with or without 25 μM C2, C6 or C16 ceramides showed an insignificant increased induction of Bax protein after 6 hrs, which was decreased after 24 hrs, but was still significantly higher than that of the untreated cells in case of C16 (Fig. 3 b and c).

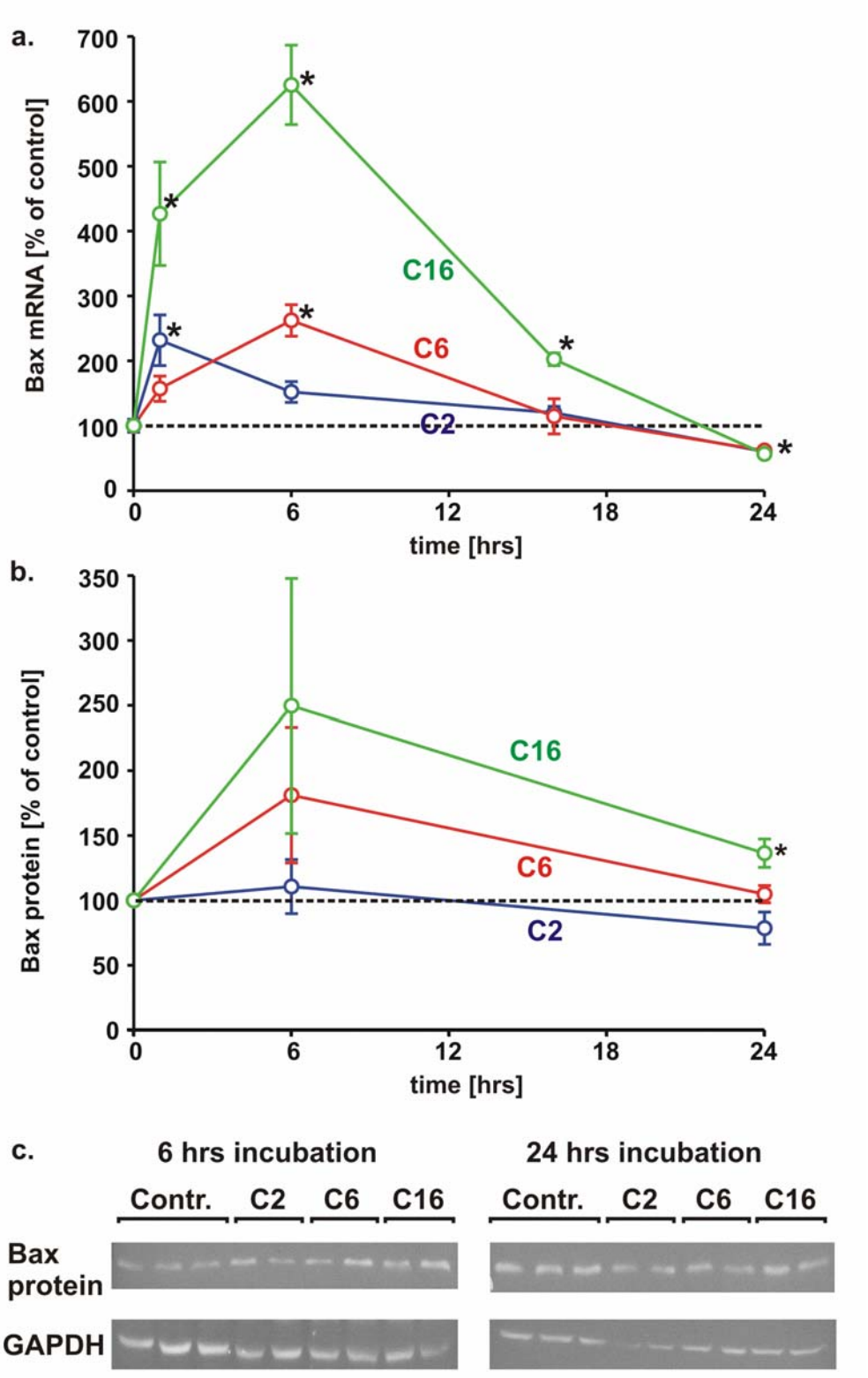

Fig. 3: Effects of ceramides on Bax protein and mRNA: H9C2 cells were incubated with 25 μM C2, C6 or C16 ceramides. (**a**) Total RNA was extracted after 1, 6, 16 or 24 hrs following ceramides treatment. Bax was detected by semi-quantitative RT-PCR as described in the materials and methods section. The data were normalized to 18S rRNA. (**b**) Quantitative data from Bax immunoblots (**c**). Total proteins were extracted after 6 and 24 hrs following ceramides incubation. The data were normalized to GAPDH. Mean values ± SEM are shown, (* $P < 0.05$).

4.2.2. Influence of ceramides on Bcl-x mRNA:

Bcl-x is a member of the Bcl-2 family that has several splice variants: the antiapoptotic Bcl-xL, which inhibits cell death, and the death inducer the proapoptotic Bcl-xS.

H9C2 cells showed a significant increase in induction of the mRNA levels of the antiapoptotic Bcl-xL in response to one hour incubation with 25 µM C2, C6 or C16 ceramides (Fig. 4 a).

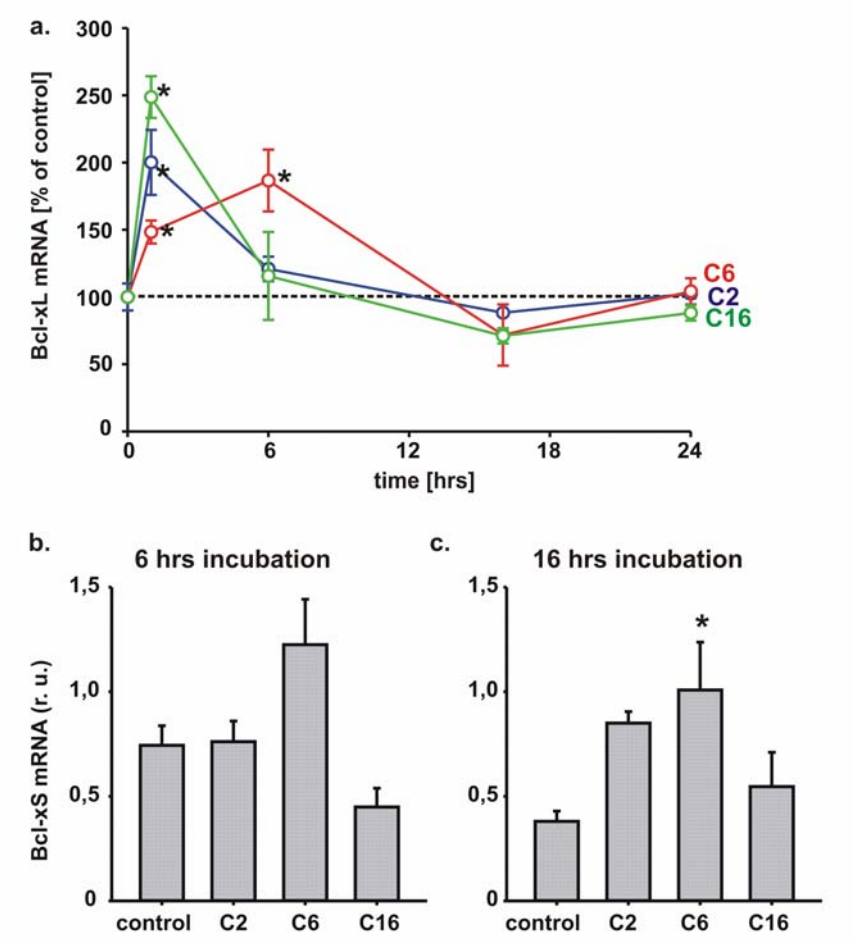

Fig. 4: Effects of ceramides on Bcl-x mRNA expression: H9C2 cells were treated with 25 µM C2, C6 or C16 ceramides. The cells were lysed and the total RNA was extracted after 1, 6, 16 or 24 hrs (**a**), 6 hrs or 16 hrs (**b**) following ceramides incubation. Bcl-xL and Bcl-xS were detected by semi-quantitative RT-PCR (**a**) and (**b**) as described in the materials and methods section, respectively. The data were normalized to 18S rRNA. Mean values ± SEM are shown, (* $P < 0.05$).

This Bcl-xL induction was significantly higher after six hrs in response to C6, then followed by insignificant reduction below the level of the controls after 16 hrs and after 24 hrs there was an insignificant increase to approach the level of the controls. In contrast, C2 and C16 showed early insignificant reduction after six hrs, but they exhibited the same pattern of C6 after 16 or 24 hrs.

The proapoptotic splice product Bcl-xS was detected after 6 or 16 hrs and was found to be significantly increased only after 16 hrs following treatment with C6 (Fig. 4 b).

4.2.3. Effects of ceramides on Bcl-xL proteins expression:

Incubation of the H9C2 cells with 25 µM C2, C6 or C16 ceramides resulted in a significant increase in the immunoreactive proteins levels of the antiapoptotic Bcl-xL by 3 - 4 times the control cells after 24 hrs, but not after 6 hrs (Fig. 5). The antiapoptotic Bcl-xS protein was below the level of detection.

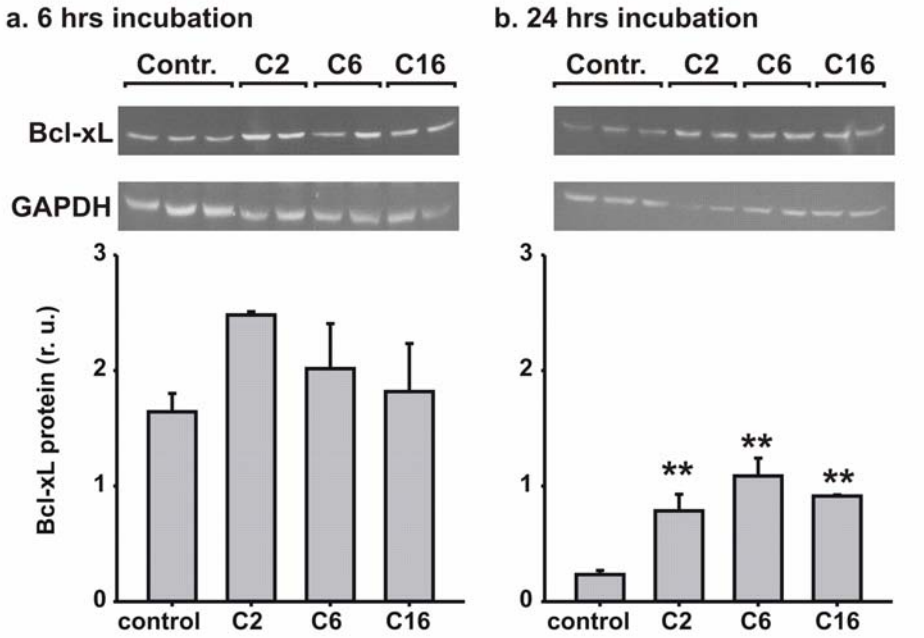

Fig. 5: Ceramides induce Bcl-xL protein expression: H9C2 cells were incubated with 25 µM C2, C6 or C16 ceramides. Total proteins were extracted after 6 hrs (**a**) and 24 hrs (**b**) following ceramides incubation. The upper panels in (**a**) and (**b**) show Western blots of Bcl-xL. The lower panels show the quantitative data from these blots. The data were normalized to GAPDH. Mean values ± SEM are shown, (* $P < 0.05$).

4.2.4. Ceramides and Bax/Bcl-xL ratio:

To determine whether ceramides affect the balance between the apoptotic and antiapoptotic members of the Bcl-2 family members, we estimated the ratio of the proapoptotic Bax mRNA to the antiapoptotic Bcl-xL mRNA (Fig. 6). Increase in this ratio may indicate a shift of the balance towards apoptosis. All the ceramides in this study increased this ratio after 1, 6 or 16 hrs. The most dramatic effects have been observed with C16 (Fig. 6). Surprisingly, this ratio was found to be

significantly reduced below the levels of the untreated cells after 24 hrs in response to the three ceramides, due to the reduced induction of Bax at this time.

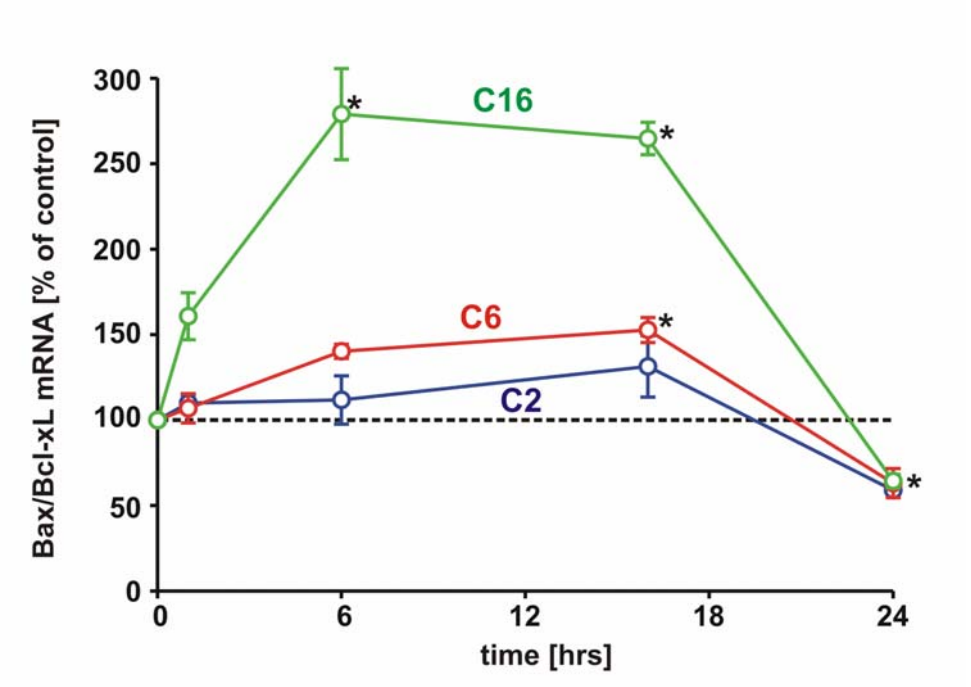

Fig. 6: Effects of ceramides on Bax/Bcl-xL ratio: H9C2 cells were treated with 25 μM C2, C6 or C16 ceramides. The cells were lysed and the total RNA was extracted after 1, 6, 16 or 24 hrs following ceramides incubation. Bcl-xL and Bax were detected by semi-quantitative RT-PCR, as described in the materials and methods section. The data were normalized to 18S rRNA. Bax/Bcl-xL ratio is shown as percentage of untreated control; increase in this ratio indicates proapoptotic shifting, while reduction indicates antiapoptotic shifting. Mean values ± SEM are shown, (* $P < 0.05$).

4.3. Influence of ceramides on cytochrome c release, caspase activation and DNA cleavage:

4.3.1. Influence of ceramides on cytochrome c release:

Cytochrome c release from the mitochondria into the cytosol is a critical step toward apoptosis. After incubation of the H9C2 cells for 24 hrs with or without treatment with 25 μM of C2, C6 or C16 ceramides, we analyzed cytochrome c in the cytosolic protein fraction by Western blot. There was found to be an increased level of cytosolic cytochrome c in response to the three different types of ceramides compared to untreated cells (Fig. 7). This effect was more prominent with C6 ceramide, whereas C16 ceramide was found to have the lowest effect.

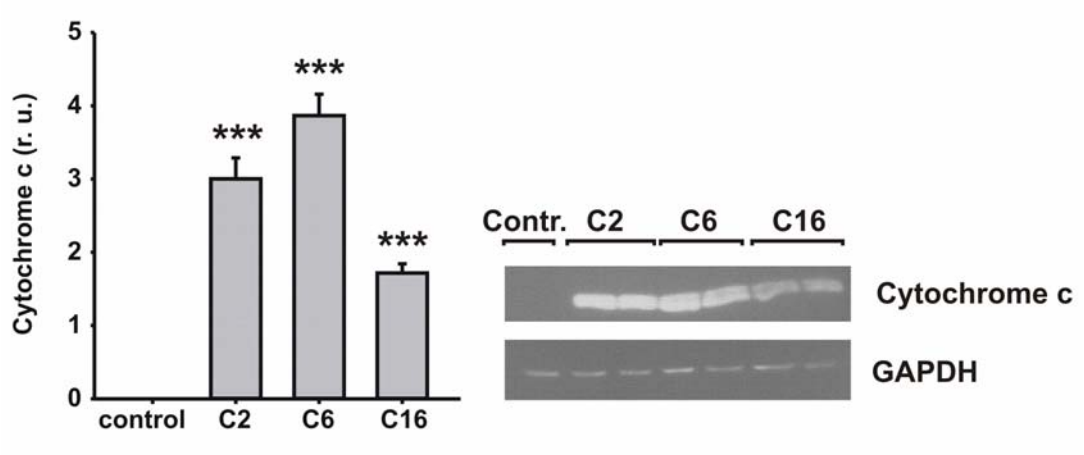

Fig. 7: Ceramides release cytochrome c: H9C2 cells were treated with 25 µM C2, C6 or C16 ceramides for 24 hrs. Cytosolic cytochrome c was detected by Western blot. The right panel is immunoblot and the left panel shows a quantitative data of this blot. There is a significant increase in the cytosolic cytochrome c following treatment with ceramides. The data were normalized to GAPDH and shown in Mean values ± SEM, (*** P<0.0001).

4.3.2. Effects of ceramides on caspase-9:

The Western blots obtained from incubation of the H9C2 cells for 6 or 24 hrs with or without 25 µM of C2, C6 or C16 ceramides showed a significant reduction of the uncleaved form of caspase-9 (Fig. 8). Although no cleavage products of caspase-9 were detected, this suggests an increased consumption of procaspase-9. The cleavage products might be unstable or broken down very fast.

Furthermore, caspase-9 activity was performed after incubation of the H9C2 cells with or without C2, C6 or C16 ceramides for 6, 16 or 24 hrs durations (Fig. 9). It was observed that C2, C6 and C16 ceramides showed a significant increase in caspase-9 activity after 6 hrs of incubation and found to be 105%, 133% and 103% of the control cells, respectively. For C2 and C16 ceramides caspase-9 activity was significantly gradually increased to reach the maximum after 24 hrs. The response of the cells to C6 ceramide incubation was found to be different: it showed a sharp increase after 6 hrs, after that continued to increase gradually to reach the maximum after 16 hrs and then decreased to reach the lowest level after 24 hrs.

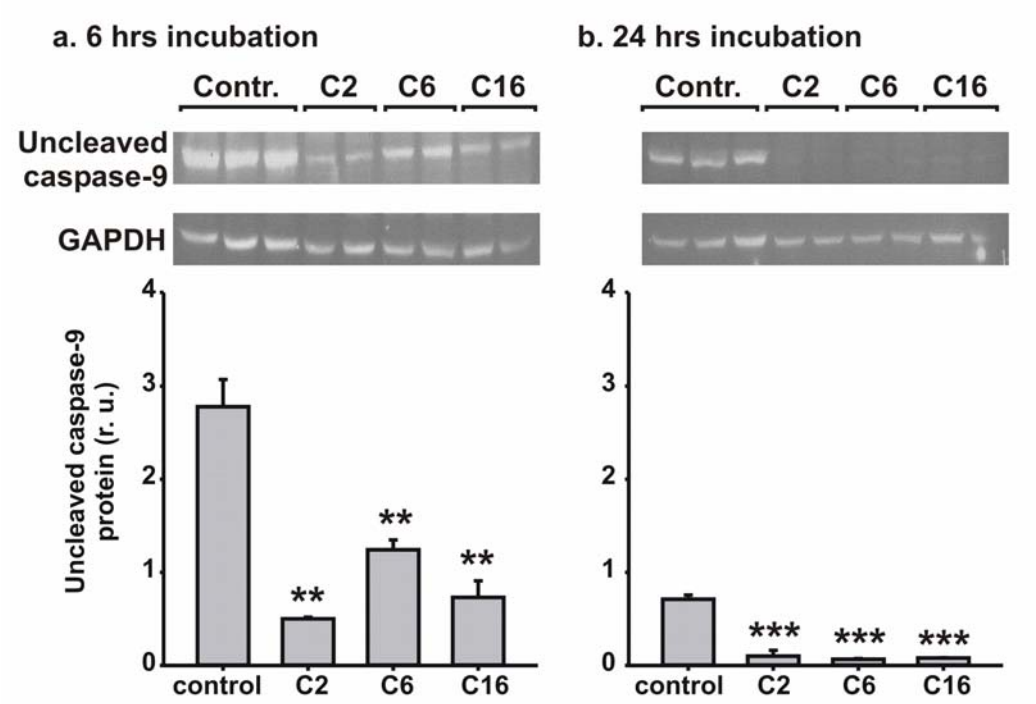

Fig. 8: Effects of ceramides on uncleaved caspase-9: H9C2 cells were treated with 25 µM C2, C6 or C16 ceramides. Total proteins were extracted after 6 hrs (**a**) and 24 hrs (**b**) following ceramides incubation. The upper panels in (**a**) and (**b**) show Western blots of uncleaved procaspase-9 and GAPDH, the lower panels show the quantitative data from the above blots. The data were normalized to GAPDH. Mean values ± SEM are shown, (** P< 0.001 and *** P<0.0001).

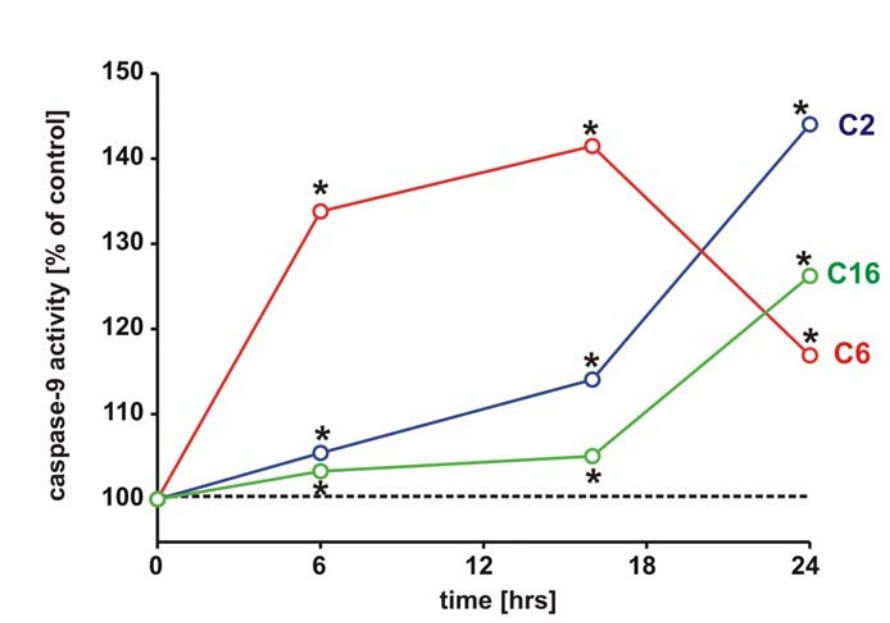

Fig. 9: Ceramides increase caspase-9 activity: H9C2 cells were treated with 25 µM C2, C6 or C16 ceramides for 6, 16, or 24 hrs. Caspase-9 activity assay was determined as described in the materials and methods section. The activity is shown as percentage of untreated control. Mean values ± SEM are shown.

4.3.3. Effects of ceramides on caspase-3 proteins levels:

Treatment of the H9C2 cells with or without 25 µM C2, C6 or C16 ceramides resulted in the reduction of procaspase-3 after 6 hrs (Fig. 10). This reduction was significant after 24 hrs, with 61%, 54% and 51% of the control cells level. Similar to the results obtained for caspase-9, we did not detect any cleaved isoform of the active caspase-3. Again this decrease in procaspase-3 may be a sign of increased consumption.

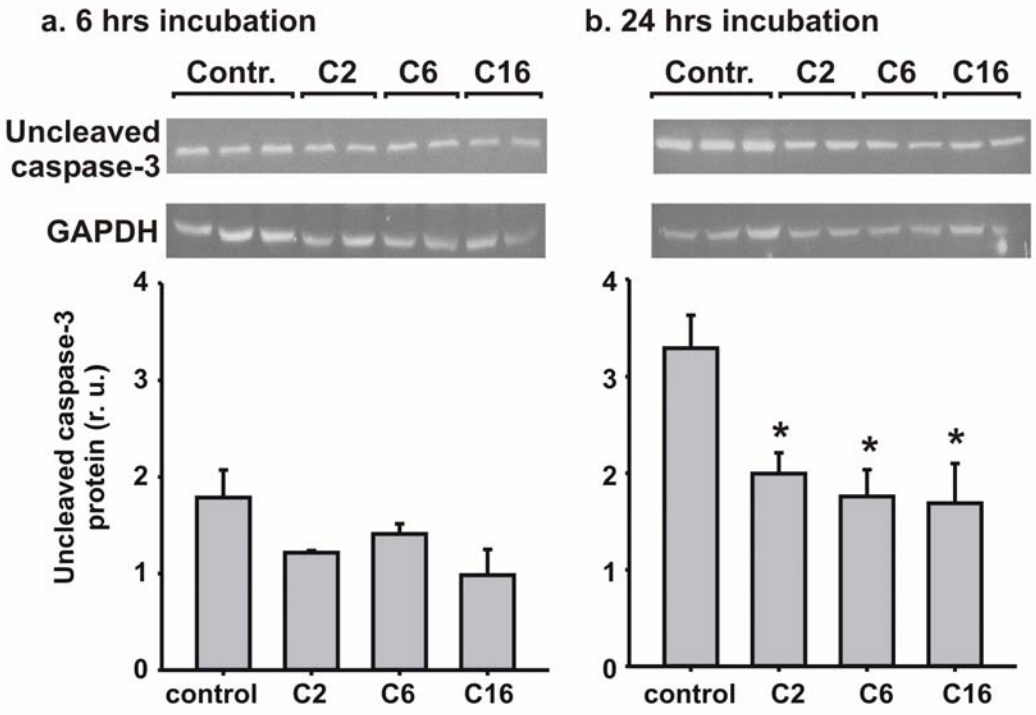

Fig. 10: Effects of ceramides on uncleaved caspase-3: H9C2 cells were treated with 25 µM C2, C6 or C16 ceramides. Total proteins were extracted after 6 hrs (**a**) and 24 hrs (**b**) following ceramides incubation. The upper panels in (**a**) and (**b**) show Western blots of uncleaved caspase-3 and GAPDH, the lower panels show the quantitative data from the above blots. The data were normalized to GAPDH. Mean values ± SEM are shown, (* $P < 0.05$).

4.3.4. Effects of ceramides on the DNA:

To determine whether the decreased number of viable cells was associated with occurrence of DNA fragmentation or not, H9C2 cells were treated with 25 µM of C2, C6 or C16 ceramides for 24 hrs. Then the extracted DNA was studied with electrophoresis. No DNA fragmentation was observed in either the control or the treated cells (Fig. 11).

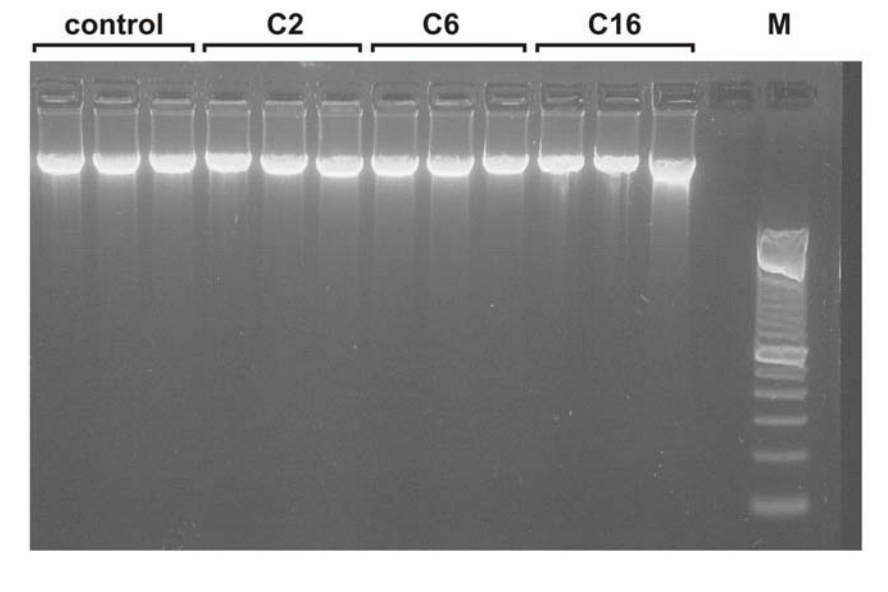

Fig. 11: Ceramides don't cause DNA fragmentation: Agarose gel electrophoresis of DNA extracted from H9C2 cells as described in the materials and methods section. The cells were treated with 25 µM C2, C6 or C16 ceramides for 24 hrs. Lane M: marker. No DNA laddering is observed.

4.4. Effects of ceramide on mitochondrial enzyme activity and mitochondrial membrane potential:

4.4.1. Effects of ceramide on mitochondrial enzyme activity:

To determine whether ceramides affect the mitochondrial enzyme activity, H9C2 cells were incubated with 25 µM of C6 ceramide for 24 hrs. After that mitochondrial enzyme activity was assessed according to the material and methods section. It was found that C6 ceramide induced a significant reduction in complex I, complex I + III, complex IV (Fig. 12). No significant effects on complex II (SDH) and complex II +III were observed.

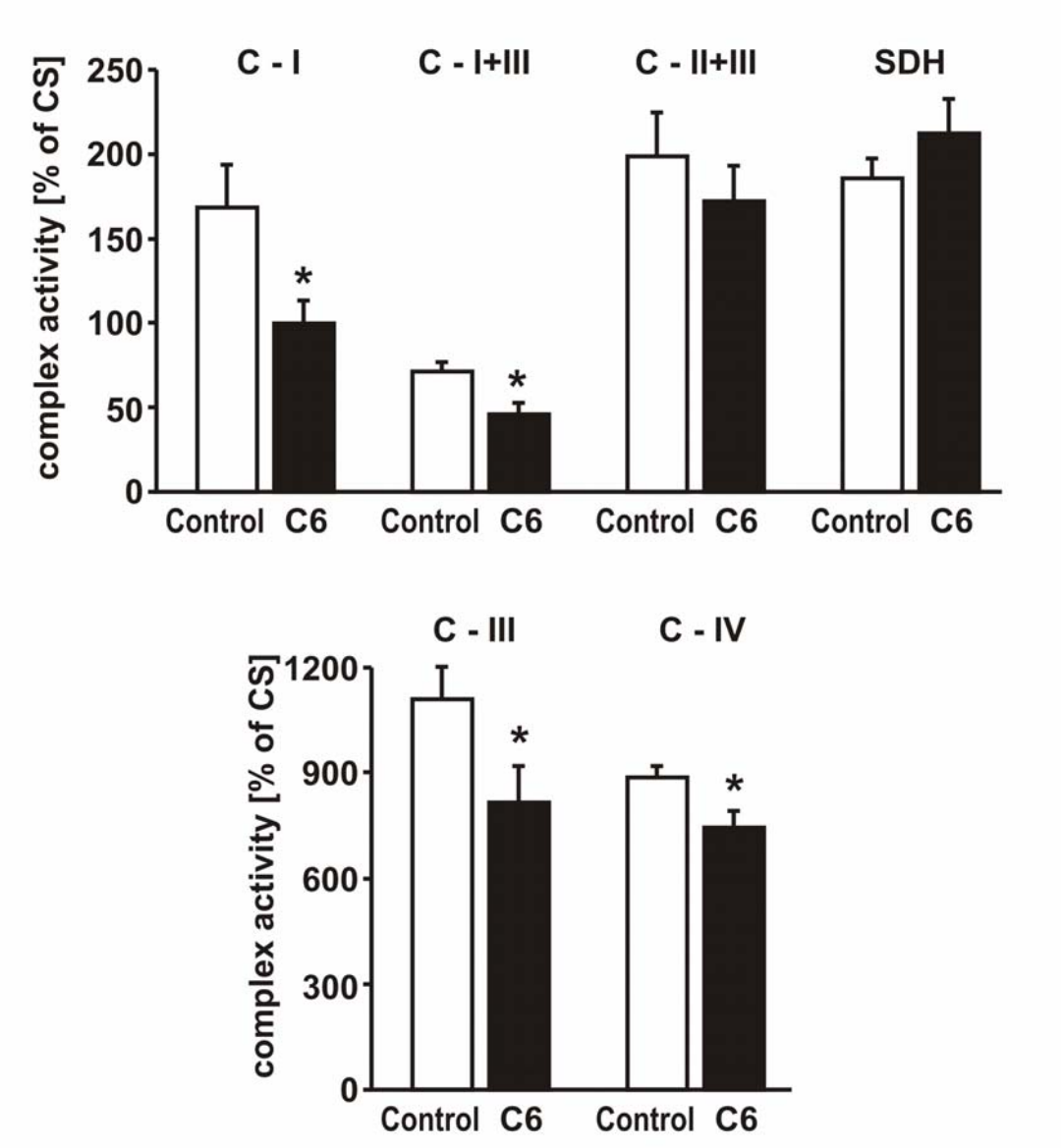

Fig. 12: Effects of ceramide on the activity of the mitochondrial respiratory enzymes: H9C2 cells were treated with 25 µM C6 ceramide for 24 hrs. The measurements of the enzymes activities were performed as described in the materials and methods section. The values are means ± SEM from three different experiments.

4.4.2. Ceramides and the mitochondrial membrane potential (ΔΨm):

JC-1 was used as an index of the electrochemical gradient across the mitochondrial inner membrane of H9C2 cells, which were incubated with or without C2, C6 or C16 ceramides for 24 hrs. The untreated H9C2 cells showed multiple red fluorescent JC-1 aggregates, consistent with a normal mitochondrial membrane potential (Fig. 13 a). In contrast, incubation of the cells with 5 µM Valinomycin, a substance know to dissipate the ΔΨm, resulted in an increase of green fluorescence emitted by JC-1 monomers, indicating a reduction of the ΔΨm

(Fig. 13 b). All the three ceramides showed a significant reduction in the ΔΨm, represented by an increase in the green florescence in relation to the red as shown in figure 13 (c-d). The quantitative data obtained from the cells counting is presented as ratio between the green and red florescence (Fig. 13 f), where increase in this ratio correlates with ΔΨm reduction.

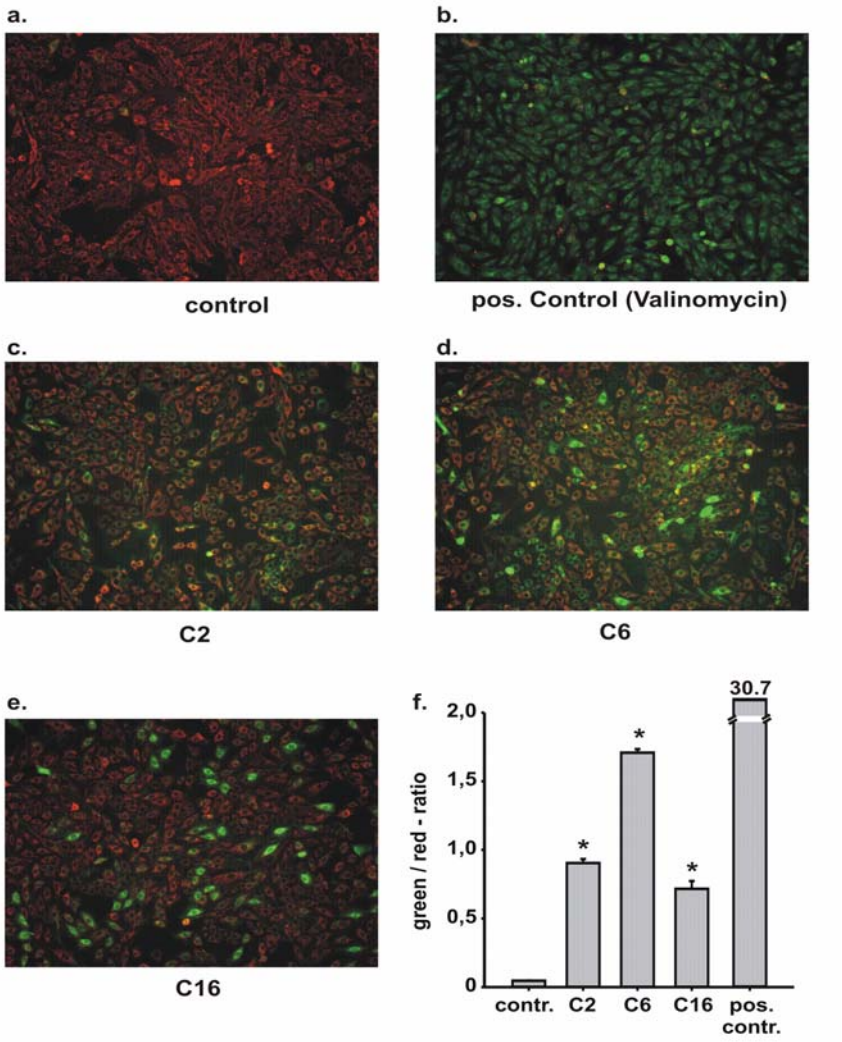

Fig. 13: Ceramides induce mitochondrial membrane potential (ΔΨm) reduction: H9C2 cells were incubated for 24 hrs in absence (**a**) or presence of 25 μM C2, C6 or C16 ceramides (**c**–**e**), respectively. ΔΨm was measured using the JC-1 dye as described in the materials and methods section. Mitochondrial depolarization is indicated by switch from the red to green fluorescence intensity. C2, C6 and C16 induced ΔΨm reduction (**c**–**e**) in comparison to the untreated controls (**a**). (**b**) The cells were treated with Valinomycin (5 μM for 10 min) as positive control. In addition, the cells with low ΔΨm were quantitated by counting them under fluorescent microscope and the data were represented as a histogram (**f**). Mean values ± SEM are shown, (* $P < 0.05$).

5. Discussion:

The various roles displayed by ceramides ranging from apoptosis to cell proliferation and gene regulation depend on the cell type, the types of activated signal transduction and the variations of the actual concentration of ceramide. Besides that, the activity of ceramide utilizing enzymes seems to have a major impact.

The present study was designed to examine the role of exogenous ceramides on the rat cardiomyoblast H9C2 cells, the subsequent mode of cell death that may occur, and the status of the balance between the promoters and inhibitors of apoptosis in this cell line.

In order to fulfil the objectives of this study, H9C2 cells were treated with the synthetic C2, C6 or C16 ceramides for different times. These cells were analysed; the cell viability, mitochondrial function and activation of apoptotic pathways were then determined.

It was found that incubation of the H9C2 cells with any of the three different ceramides for 24 hrs resulted in a reduction of the cell viability. C6 ceramide was found to have a more prominent effect when compared to C2 and C16 ceramides. It was also found that treatment with C2, C6 or C16 ceramides caused significant reductions in the mitochondrial membrane potential ($\Delta\Psi m$) and significantly increased cytochrome c in the cytosolic protein fraction. In addition, treatment of the cells with C6 ceramide also caused a significant reduction in mitochondrial complex I, complex I+III, complex III and complex IV activities. Furthermore, after one hour of treatment of the cells with any of the three different types of ceramides, there was an induction of Bax mRNA, but surprisingly there was a significant reduction in Bax mRNA after 24 hrs. The three ceramides also caused an increased induction of Bcl-xL, with a statistically insignificant Bcl-xS splicing observed after 6 and 16 hrs following treatment with ceramides. In addition, there was an increased caspase-9 activity following treatment with the three ceramides, and decreased protein expression of the uncleaved caspase-3.

Since cell death is generally classified as necrosis or apoptosis on the basis of established morphological and biochemical criteria, our findings of reduced cell viability led us to investigate the type of cell death which resulted from ceramides treatment. Classically, necrosis is considered as a passive collapse of

homeostasis and apoptosis as an active process. Nevertheless, it should be noted that increasing evidence suggests that the two forms of cell death may also share similar characteristics at least in the signalling and early execution events [113]. Our data demonstrate a mode of programmed cell death exerted by ceramides in H9C2 cells, which does not fit with the classical features of apoptosis. Therefore, it may be described as "atypical apoptosis".

5.1. Mode of ceramide application:

In the present study, H9C2 cells were treated with synthetic C2, C6 or C16 ceramides. Exogenous application of ceramides was used because naturally occurring ceramides, generated within the cell and with extremely hydrophobic nature, cannot be introduced into the cell without substantial alterations in cellular metabolism. However, questions have been raised regarding the significance of findings resulting from the use of exogenous ceramides. Synthetic short chain C2 and C6 ceramides can cross the cell membranes, but they are not natural and have been shown to induce cytochrome c release and apoptosis when added to whole cell cultures or isolated mitochondria [114, 115]. In addition, long chain naturally occurring C16 ceramide has substantially lower membrane permeability and has also been shown to induce cytochrome c release from isolated mitochondrial suspension [94]. All these three ceramides are discussed in this study as a group, but we have to consider that in whole cell culture C16 may exert its effects on the mitochondria indirectly, at least in part, since its cellular permeability is rather low, while C2 and C6 can cross the cell membrane and act directly.

However, it was observed that treatments of cells with these synthetic ceramides have similar effects to those obtained from intracellular accumulation of the endogenous ceramide [116].

5.2. Effects of ceramides on cell survival:

H9C2 cells were treated with C2, C6 or C16 ceramides in order to study their effects on the cell viability after 24 hrs.

Cell viability was determined by the MTT assay, which revealed that addition of exogenous C2, C6 or C16 ceramides to H9C2 cells in culture caused a loss of cell viability in a time and concentration dependent manner (Fig. 2). The magnitude of cell death was comparable between the three ceramides in concentrations below 3.2 μM. The cell viability varied with higher concentrations and was found to be 71%, 63% and 78% of the untreated control cells for C2, C6 and C16 at 25 μM ceramides concentration, respectively. When the concentration of ceramides was increased above this value, the behaviour of the cells was dramatically different between C6 and the other two ceramides: the viability of the cells dropped rapidly to reach 9% of untreated control cells within 24 hrs of treatment of the cells with 50 μM C6 ceramide. It reached 46% and 47% of viable cells after treatment with 50 μM of C2 and C16 ceramides, respectively. It appears that the viability of H9C2 cells in response to ceramides exposure is not directly related to the acyl chain length of the ceramides in our experiments. However, Arora and co-workers found that in rat hepatocytes the effects of ceramides on the cell viability were related to the acyl chain length of ceramide. C6 ceramide showed more reduction of cell viability than C2, while the maximal reduction in cell viability was observed with C8 ceramide. C16 was not included in their study [117].

5.3. Effects of ceramides on the patterns of the Bcl-2 family members:

Members of the Bcl-2 family, which include proapoptotic proteins and death-suppressors, are expressed to varying degrees in the different cell types, indicating that cells have distinct pathways by which death signals can be routed [118].

In this study, we investigated Bax as a typical proapoptotic protein of the Bcl-2 family that plays an important role in mediating cell death under both physiological and pathological conditions. Ablation of Bax can attenuate cardiac damage through inhibition of cardiac cells apoptosis, which signifies the importance of this protein in the regulation of cardiac cells survival [119]. Moreover, translocation of endogenous Bax from the cytosol to the mitochondria was found to be an important step during apoptosis of H9C2 cardiomyoblast cells following hypoxia-reoxygenation, serum withdrawal, menadione treatment and a number of other stimuli [120].

In addition to Bax, the antiapoptotic protein Bcl-xL and its proapoptotic splice variant Bcl-xS were also investigated, since it was reported that Bcl-xL did block the function of Bax by the inhibition of its translocation from the cytosol to the mitochondria [25, 121]. In this investigation, we used semiquantitative RT-PCR and Western blots of the Bcl-2 family members in the H9C2 cells under C2, C6 or C16 ceramides exposure.

An induction of Bax mRNA was found after one hour following treatment with the three types of ceramides, which was significant for C2 and C16 ceramides (approximately 2 and 4 times of the untreated controls, respectively) (Fig. 3a). Induction of Bax in response to C6 and C16 ceramides significantly continued to increase until six hrs of treatment then started to decline towards the levels of the controls after 16 hrs. However, the effect of C16 on Bax mRNA was still significant and two times higher than the controls after 16 hrs. In case of C2, the reduction of Bax mRNA started before six hrs. After 16 hrs, the level of Bax mRNA expression dropped below the control level. This was also significantly seen after 24 hrs of treatment with all three types of ceramides. These findings from the RNA were comparable with the protein expression, as shown in the quantitative data obtained from the immunoblots (Fig. 3b and c). It was also found that Bax protein increased after six hrs and then tended to decrease to reach the lowest levels after 24 hrs for all three ceramides. However, these values were still significantly higher than those of the untreated control cells in case of treatment with C16. From these findings, it is obvious that there was an early induction of Bax mRNA and protein. Probably, the most affected cells that have highest expression of Bax were lost, while the cells with minimal Bax expression survived. Therefore, this may explain the later reduction of Bax level on both the protein and mRNA levels. As described by Kim et al., C2 ceramide was found to be able to induce Bax expression in mouse mammary gland cells (HC11) after 3 hrs and this induction was found to be significantly declined after 36 hrs of treatment [26]. However, no data are available for C6 or C16 ceramides in the literature and no comparable study was performed in muscular cells.

Furthermore, the antiapoptotic Bcl-xL was found to be significantly induced in the H9C2 cells to 200%, 148% and 248% of the untreated control values in the first hour of treatment in response to C2, C6 or C16 ceramides, respectively (Fig. 4a). This effect was more prominent following treatment with C16. Induction of Bcl-xL

following C2 and C16 was reduced after the first hour to reach levels below that of the control values after 16 hrs of treatment. On the other hand, induction of Bcl-xL produced by C6 ceramide continued significantly to be higher than the control values until 6 hrs of treatment and after that it was reduced in the same way as C2 and C16. After 24 hrs of treatment, the three ceramides showed increased induction of Bcl-xL, which was also significantly seen in the protein expression (Fig. 5b).

Statistically insignificant alteration in splicing towards the Bcl-xS, the apoptotic isoform of the Bcl-x, was seen after 6 and 16 hrs following treatment of the H9C2 cells with C2, C6 or C16 ceramides (Fig. 4b and c). This splicing effect was not detectable in immunoblots obtained after 6 and 24 hrs following treatment with C2, C6 or C16 ceramides (Fig. 5a and b), most likely the amount of Bcl-xS protein was very low and therefore below the level of detection. Interestingly, only one molecule of Bcl-xS can overcome the antiapoptotic effects of four molecules of Bcl-xL. This reflects the efficacy of the Bcl-xS proteins to counteract the antiapoptotic function of Bcl-xL [35]. Therefore, the balance between the proteins amounts of these pro- and antiapoptotic isoforms of the Bcl-x gene can decide whether the cells undergo apoptosis or not. Moreover, the alternative splicing of the Bcl-x gene in response to ceramide treatment was known to be time and dose dependent phenomenon. In the lung cancer cell line A549 C6 ceramide did not affect the alternative splicing of the Bcl-x gene until after 16 hrs of treatment, with maximal effects seen after 36 hrs [34]. It appears that this system of ceramide-induced shifting in Bcl-x splicing at this concentration of ceramides and under the present experimental conditions is different in H9C2 cells compared to A549. This Bcl-x shifting under ceramide influence has not yet been analysed before in these cells. Other Bcl-x splice variants such as Bcl-xβ may be involved, but were not investigated in this study.

It is generally believed that the ratio of death agonists, such as Bax and Bcl-xS and death antagonists, such as Bcl-2 and Bcl-xL plays a major role in the fate of the cells following an apoptotic stimulus [122]. Our model demonstrated an increase in Bax and a decrease in Bcl-xL, resulting in a significant increased proapoptotic ratio of Bax to Bcl-xL seen following 6 and 16 hrs treatment with C16 ceramide and after 16 hrs with C6 (Fig. 6). This proapoptotic ratio suggests strongly that apoptosis did play a role in initiation of cell death during these time

points. In spite of the reduction of the cell viability, this proapoptotic ratio was significantly decreased at 24 hrs due to the increase in Bcl-xL induction. In H9C2 cells, the induction of the antiapoptotic Bcl-xL in response to treatment with ceramides seems to be a protective mechanism from the diverse cytotoxic effects of ceramides, but it failed to stop the cell loss. This may suggest that apoptosis due to an imbalance between the apoptotic and antiapoptotic factors was not the only cause of reduction of the cell viability. However, several other studies in various models did show that the increase in the ratio of Bax and Bcl-xL was indicative of an increase in apoptotic death [123, 124].

Furthermore, in response to C2 ceramide treatment, the mouse mammary cells HC11 also showed an induction of the antiapoptotic Bcl-2 in the first 3 hrs of treatment and then a return to the basal level at 6 and 12 hrs. Then it increased again to reach the maximum induction at 24 hrs [26]. In another study, H9C2 cells were found to respond to doxorubicin, which is a known cytotoxic agent and apoptotic inducer, by induction of the antiapoptotic Bcl-2 protein [123].

5.4. Ceramides release cytochrome c:

Cytochrome c is a diffusible component of the mitochondrial respiratory chain, normally located in the intermembrane space of the mitochondria. Concomitantly, it is an apoptogenic protein, when released from the mitochondrial intermembrane space into the cytosol. In general, this release is important for caspase activation during the terminal steps of apoptosis as well as in cardiac lipoapoptosis [14, 15, 125, 126]. In this study, cytosolic cytochrome c was found to be significantly elevated in H9C2 cells following 24 hrs treatment with C2, C6, or C16 ceramides, rendering the cells vulnerable to the proapoptotic conditions (Fig. 7). The cytosolic cytochrome c was found to be more prominent with C6 and relatively lower in C16 ceramide. In comparison, Di Paula and co-workers also found that both C2 and C16 ceramides induced cytochrome c release from isolated rat heart mitochondria. However, in their study the directly acting C16 showed stronger effects [94]. The increased permeability of the mitochondrial membrane for cytochrome c in the present study may be explained by the over expression of Bax, since Bax was found to cause mitochondrial conformational changes and the release of cytochrome c [24, 92]. Another possibility for the increased permeability of the

mitochondria is the direct physicochemical effects of the short chain ceramides on the mitochondria. The direct effects exerted by ceramides on the mitochondria may be earlier events, while the effects of Bax are additional.

5.5. Effects of ceramides on caspase-9 and 3:

Caspases are a group of cysteine proteases involved in the execution of apoptosis. Based on their functions during the apoptotic process, they are divided into two groups: initiator and effector caspases. Caspase–3 is considered to be the main effector caspase, inducing the fundamental features of apoptotic cell death. However, it was demonstrated that mice with deleted caspase-3 gene have effective apoptotic mechanisms [127, 128].

The release of cytochrome c from mitochondria is a crucial step in the activation of the caspase cascade. We found that C2, C6 and C16 ceramides significantly reduced the level of the uncleaved procaspase-9 from the Western blot analysis after 6 hrs or 24 hrs following incubation of the cells with ceramides (Fig. 8). These findings were supported by the results obtained from caspase-9 activity assay, which showed a significant increase in caspase-9 activity following treatment of H9C2 cells with ceramides (Fig. 9). It was observed that, after six hrs of treatment C2 and C16 ceramides increased caspase–9 activity to 105% and 103% of that of the untreated controls, respectively. This effect was gradually increasing to reach the maximum levels after 24 hrs, and the effect of C2 was still higher than that of C16, 144% and 126% of the untreated controls, respectively. In contrast, C6 ceramide showed a different pattern; it induced a sharp increase in caspase-9 activity after six hrs to reach a peak after 16 hrs and then followed by reduction to reach the lowest level after 24 hrs. Thus, the response of H9C2 cells to treatment with ceramides showed a pattern of caspase-9 activity that varies according to the type of ceramide that was used and the duration of treatment of the cells with ceramides.

The Western blot analysis of caspase-3 also showed a significant reduction of the uncleaved procaspase-3 after 24 hrs following treatment with the three ceramides, but no activated cleaved form of caspase-3 was detected (Fig. 10). The three ceramides showed almost the same degree of uncleaved caspase-3 reduction. Ceramide was reported to cause apoptosis in A549 lung adenocarcinoma cells,

depending on the activation of caspase-9 and 3 [129]. In contrast, treatment of A172 human glioma cells with C2 ceramide was found to induce cell death without altering caspase-3 activity, indicating that ceramide can induce programmed cell death through caspase independent mechanisms [130]. However, an increasing number of reports have suggested the existence of caspase independent programmed cell death with some necrotic features that can be regulated by genetic manipulation of the survival signalling pathway, which is sometimes called non apoptotic programmed cell death or atypical apoptosis [131]. Although our findings suggest that the activation of caspase-3 may be involved in the reduction of H9C2 cell survival, which was induced by ceramide, it is still possible that the processing of caspase-3 is only an accompanying or a subsequent event after apoptosis has already been triggered, rather than an essential step for induction of apoptosis.

5.6. Ceramides and the DNA laddering:

Although different techniques for the isolation of DNA or the detection of small amounts of DNA-strand breaks were applied, we did not observe any DNA laddering of the extracted DNA after agarose gel electrophoresis, following treatment of H9C2 cells with C2, C6, or C16 ceramides (Fig. 11).
However, according to Collins et al. [132], evidence of DNA laddering is not a definitive hallmark of apoptosis and should not be used as a sole criterion for apoptosis, since internucleosomal fragmentation can also occur as a result of necrosis and many nuclei must be caught in a synchronous fragmentation patterns to get a result. On the other hand, Krown and co-workers reported DNA fragmentation following treatment of neonatal ventricular cardiomyocytes with C2 ceramide [83].

5.7. Ceramides and mitochondrial enzyme activities:

The interactions of ceramide with the respiratory chain components may occur at various levels. In the present study we found that, C6 ceramide caused inhibition of NADH-ubiquinone reductase (complex I), NADH-cytochrome c reductase (complex I+III), ubiquinol-cytochrome c reductase (complex III) and cytochrome c

oxidase (complex IV) activities in H9C2 cells. The strongest effect was observed in complex I (Fig. 12). This was not accompanied by a comparable inhibition of succinate dehydrogenase (complex II) and succinate-cytochrome c reductase (complex II +III) activities. These inhibitory effects of ceramide on mitochondrial enzyme activities can be caused directly by cytochrome c release in case of complex I or complex I+III, and also due to indirect alteration in the respiratory chain secondary to cytochrome c loss concerning complex III or complex IV.

In isolated rat heart mitochondria, Di Paula and co-workers found that C2 caused a significant stimulation of complex IV, whereas C16 caused an opposite effect, which is inhibition of the enzyme activity. In the same study they found that the activities of both complex I and complex I+III were inhibited by C2 and C16, while complex II+III and complex III were unaffected by ceramide [94]. However, in their study, isolated mitochondria have been preselected by the process of differential centrifugation. Damaged mitochondria are more susceptible to the effects of substances like ceramides are removed during the centrifugation process. Therefore, the results from isolated mitochondria are not comparable to the results from cell lysates.

In another study, it was found that low doses of C6 ceramide were very effective in inhibition of complex III activity in rat heart mitochondria, whereas higher doses did not produce further reduction of complex III activity, which is a possible direct target for ceramide in the cells [97].

Nevertheless, mitochondrial respiration dysfunction may occur much earlier than the appearance of classical features of either apoptosis or necrosis. It is obvious from this discussion that there is still debate on the effects of ceramides on the mitochondrial function, and the exact mechanisms through which ceramides induce these effects are remaining controversial. However, solving the problem of controlling the mitochondrial respiration in order to enhance apoptosis, may contribute to optimize the therapeutic strategies in the treatment of human cancers and to decrease the inflammatory processes induced by necrosis.

5.8. Influence of ceramides on the mitochondrial membrane potential (ΔΨm):

Disruption of the ΔΨm was known to be involved in ceramide-induced apoptosis in human cutaneous T-lymphoma cells Hut79 [133]. From the above discussion it

can be expected that the interaction of ceramides with the mitochondria might affect the ΔΨm by several possible mechanisms. These include release of cytochrome c, which is known to have a critical role in stabilisation of the ΔΨm, into the cytosol following ceramides treatment, the inhibitory effects of C6 ceramide on complex I, complex III and complex IV (cox) activities or finally the physicochemical properties of ceramides which may alter the membrane permeability [134].

In this study, H9C2 cells showed a significant reduction in the ΔΨm following incubation with the three ceramides. The effect of C6 was higher compared to C2, whereas C16 showed the lowest effect (Fig. 13). In contrast to our findings, Macro Di Paola and co-workers found that in isolated mitochondria from rat ventricular cardiomyocytes, C6 had negligible effect on the mitochondrial membrane potential, while C2 caused a significant drop in it [94]. Generally, it can be observed that C6 ceramide exhibited more toxic effects on our model, while C16 showed the lowest effects. This may indicate that C16 does not reach the mitochondria directly.

5.9. Future strategy:

According to the presented data in this study, H9C2 cells showed in response to treatment with C2, C6 or C16 ceramides atypical apoptosis. Furthermore, the cells showed only marginal insignificant alteration in the Bcl-x splicing following ceramides treatment.

Therefore, these findings make this model of ceramide-induced loss of viability not ideal for investigation of the apoptotic shifting of the Bcl-x splicing, which was observed in the left ventricle from aging rats hearts. Nevertheless, this does not exclude the possibility of an involvement of accumulation of endogenous ceramide in the proapoptotic shifting in the aging rats hearts. Treatment of the cells in this study with the exogenous short chain cell permeable ceramides like C2 and C6 was found to mimic some of the effects of the natural ceramides in different studies, but still these ceramides are not physiological, and they may exert different effects directly on the mitochondria. Moreover, the long chain C16 ceramide was observed to cause direct effects on isolated mitochondria, but the effect on the whole cell culture may also be different from the naturally

accumulating ceramide and still we are not sure if it enters the cell or not. Therefore, intracellular ceramide measurement should be performed to solve this problem. Our findings also may indicate that the immortalized H9C2 cells are not ideal cells to mimic the effects of ceramides on adult cardiomyocytes. In contrast, adult cardiomyocytes themselves also may not be ideal, since they undergo spontaneous apoptosis and dedifferentiate easily in culture.

The problem of addition of exogenous ceramides can be overcome by pharmacological manipulation of the endogenous ceramide, either by using ceramide agonists, which induce ceramide production like sphingomyelinases or inhibitors of ceramide breakdown. These treatments also should be handled with care, since they can result in accumulation of ceramide, which may exceed the physiological doses.

Incorporation of the naturally and more physiologically relevant C16 ceramide into a liposome may facilitate its entry into the cells. This procedure may ensure direct and controlled delivery of ceramides in the cells. However, this controlled delivery by liposomes may be difficult in case of the short chain ceramides, because of their rapid exchange between lipid membranes.

Moreover, in vivo models will ultimately be required to test the hypothesis of ceramide-induced proapoptotic alteration in Bcl-x splicing in aging hearts and to elucidate the role of ceramides in cardiomyocytes functions.

5.10. Ceramides and cancer therapy:

Increasing levels of ceramide may be the basis for many diseases as well as aging, but it is also responsible for a major mechanism to arrest tumor growth and induce cell death in tumor cells. A number of clinically important cytotoxic agents appear to be effective due to their ability to activate ceramide-mediated pathways in cancer cells. Drugs can impact the ceramide metabolism in many different ways, such as promoting ceramide synthesis de novo like etoposide and anthracycline [135], by activating sphingomyelinase like ionizing radiation [55, 68], or by blocking glucosylceramide formation like tamoxifen [68, 136].

Furthermore, treatment of A549 cells (lung cancer cell line) with the chemotherapeutic agent gemcitabine was found to increase endogenous ceramide synthesis via de novo pathway and at the same time down regulate the level of

Bcl-xL mRNA with concomitant increase in Bcl-xS mRNA via alternative splicing. Moreover, this ceramide-induced alteration in the splicing of the Bcl-x gene was found to increase the sensitivity of these cells to daunorubicin [34]. Ceramides are involved in mechanisms that mediate the alternative splicing of many key apoptotic regulators such as Fas, caspase-2, caspase-9 or Bcl-x. Therefore, manipulation of these splice variants may be a useful tool in chemotherapy. There are already studies showing that the use of oligonucleotide that specifically interacted and blocked the 5' splice site for Bcl-x L in A549 cells induced apoptosis and increased the sensitivity of these cells for daunorubicin [137-139]. Thus, an interference with the alternative splicing of Bcl-x or other key apoptotic regulators may make tumor cells more succeptible to undergo apoptosis and therefore represent a new therapeutic concept to treat various types of tumors by increasing their sensitivity against classical chemotheropeutic agents.

However, some anticancer drugs, like anthracycline, ionizing radiation and doxorubicin are also known to induce irreversible myocardial injury and endothelial cell death [61, 140]. Therefore, selective ceramide elevation and increasing its toxicity toward malignant but not normal cells would be preferable to protect the healthy tissues from the lethal effects of cancer therapy. However, no such drugs are available so far and therefore a detailed history of drug exposure in patients with signs or symptoms of heart failure is mandatory, since numerous drugs from various drug classes may precipitate or worsen heart failure.

6. Summary:

Ceramide is a membrane sphingolipid that has been described as a lipid second messenger. It can be generated within the cell de novo or by hydrolysis of sphingomyelin upon stimulation of several sphingomyelinases. Ceramide formation serves many different functions at distinct locations in the cell and it is involved in various signalling pathways implicated in cell cycle arrest, differentiation or senescence. Moreover, evidence is accumulating pointing to the critical roles of ceramide in the induction of apoptosis. Ceramide is directly involved in stress stimuli-induced, growth factor-deprivation mediated or receptor-mediated apoptosis. Besides that, it is also involved in mechanisms that mediate the alternative splicing of many key apoptotic regulators, such as Fas, caspase-2, caspase-9 or Bcl-x.

In the current study, the effects of the cell-permeable C2 and C6 ceramides as well as the long-chain C16 ceramide on H9C2 cells viability, induction of cell death, mitochondrial function and the expressional patterns of members of the Bcl-2 gene family, which are known as modulator of mitochondrially mediated cell death, were analyzed.

All the three ceramides induced a significant reduction of H9C2 cells viability after treatment for 24 hrs and C6 was found to be the most toxic. H9C2 cells also showed a significant reduction in the mitochondrial membrane potential, in response to incubation with any of the three ceramides. In addition, C6 significantly reduced mitochondrial complex I, complex I+III, complex III and complex IV activities. A significant induction of the proapoptotic Bcl-2 family member Bax was observed after one hour of ceramides treatment, but decreased after 24 hrs to levels below the untreated controls. Interestingly, the antiapoptotic isoform of the Bcl-x gene, Bcl-xL, was increased as well as the proapoptotic isoform Bcl-xS following treatment with any of the three ceramides. Moreover, all the three ceramides caused an increased caspase-9 activity in H9C2 cells and a reduction in both uncleaved procaspase-3 and 9, most probably due to the increased consumption of procaspase-3 and 9. At the same time, the three ceramides also increased cytochrome c release from the mitochondria into the cytosol as a sign of the mitochondrial mediated apoptosis, but no DNA cleavage was detectable in any of the samples.

In summary, exposure of H9C2 to the exogenous C2, C6 or C16 ceramides resulted in mitochondrial dysfunction and in a programmed cell death with atypical features of apoptosis. Therefore, H9C2 cells may be a suitable cellular model to study only certain aspects of ceramide-mediated pathways in the ageing myocardium. Further studies of the molecular basis of these phospholipids may facilitate advances in the discovery of drugs, which mitigate or even prevent diseases that may result from an elevation in ceramide level.

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8. Theses:

1. Apoptosis and necrosis are well known causes of the loss of cardiomyocytes in aging heart, a phenomenon which may contribute to the myocardial dysfunction that occurs in the elderly.

2. Necrosis is considered as a passive collapse of homeostasis and apoptosis as an active process, but it should be noted that increasing evidence suggests that the two forms of cell death may also share similar characteristics at least in the signalling and early execution events.

3. A significant shift towards the proapoptotic isoform of the Bcl-x gene (Bcl-xS) is observed in aging hearts, and this may render cardiomyocytes more susceptible for apoptosis induced by several stimuli and is associated with mitochondrial dysfunction.

4. The proapoptotic shift in Bcl-x splice products in the aging myocardium can be corrected back to the level of the normal value of young animals by a moderate transient caloric restriction.

5. Ceramide can cause shifting in various apoptosis related genes such as Fas, Bcl-x, caspase-2 and caspase-9. This mechanism may also play an important role in the aging heart.

6. Ceramides are sphingolipids that have been described as messengers for several events like differentiation, senescence, proliferation, apoptosis and cell cycle arrest in indifferent cell lines.

7. Ceramide can be formed through sphingomyelinases-dependent catabolism of sphingomylin, as well as by de novo synthesis.

8. Incubation of H9C2 cardiomyoblasts with C2, C6 or C16 ceramides results in a significant reduction in cell viability.

9. All the three ceramides show a reduction in the mitochondrial membrane potential, and an increased cytosolic cytochrome c as a sign of mitochondrial induced apoptosis in H9C2 cells.

10. Treatment of the H9C2 cells with C6 ceramide results in a significant reduction of mitochondrial complex I, complex I+III, complex III and complex IV activities.

11. H9C2 cells show a significant induction of the proapoptotic Bcl-2 family member Bax and also an increased induction of the antiapoptotic Bcl-xL with an insignificant splicing towards the proapoptotic Bcl-xS in response to ceramides treatment.

12. In response to incubation of H9C2 cells with any of the three ceramides, there is increased caspase-9 activity and decreased uncleaved procaspase-3 and 9.

13. There is no DNA fragmentation observed in response to any of the three ceramides.

14. Incubation of H9C2 cells with C2, C6 or C16 ceramides results in a programmed cell death which does not fit with the classical features of apoptosis, and it may be described as "atypical apoptosis".

15. In vivo models are ultimately required to test the hypothesis of ceramide-induced proapoptotic alteration in Bcl-x splicing in aging hearts and to elucidate the roles of ceramide in cardiomyocytes functions.

Curriculum Vitae

Name: Amir Mohmed Ahmed Abushouk

Date of Birth: 26th July 1969.

Place of Birth: Eldain – Sudan.

Nationality: Sudanese.

Marital Status: Married with two children.

E.mail Address: amirabushouk@yahoo.com

Qualifications:

- M.B.B.S. Faculty of Medicine, University of Khartoum, Sudan, 1995.
- MSc. Physiology, Faculty of Medicine, University of Khartoum, 2000.

Posts held:

- **Jul. 1997 – Jul. 2000:** Teaching Assistant, Department of Physiology, Faculty of Medicine, University of Khartoum.
- **Aug. 2000 –Sep. 2003:** Lecturer, Department of Physiology, Faculty of Medicine, University of Khartoum.
- **Since Oct. 2003:** DAAD Fellowship.

Experience:

- **Nov. 1995 – Aug. 1997:** House officer, Federal Ministry of health, Sudan, as follows:
  - Nov. 1995 - Mar 1996, Surgery, Soba University Hospital.
  - Apr. 1996 - Jul.1996, Paediatrics, Soba University Hospital.
  - Aug.1996 - Nov. 1996, Medicine, Soba University Hospital.
  - Dec. 1996 - Mar. 1997, Obstetrics & Gynaecology, Singa Teaching Hospital.

    - Apr. 1997- Jun. 1997, Orthopaedics Surgery, Central Police Hospital.
    - Jul. 1997- Aug 1997, Urology, Khartoum teaching Hospital.
- **Sep.1997- Mar.1998:** Medical Officer, Ibrahim Mallik Hospital, Khartoum, Sudan.

Researches:

- Local Hyperhidrosis in Mycetoma, Feb 2000, MSc. thesis, Faculty of Medicine, University of Khartoum.
- Malnutrition in a displaced community at Eldain, Southern Dar fur, Sudan. “A study of nutritional status in children below five years”. (unpublished).
- Effects of ceramide on cardiomyoblast viability and mitochondrial function.

Activities:

- Member of medical students’ charity society.
- Member of Sudan Doctors Union.
- Registered member of Sudan Medical Council.
- Member of Sudan Physiological Science Society.
- Student membership of the American Physiological Society (APS).

Eigenständigkeitserklärung

Hiermit erkläre ich, dass die vorliegende Arbeit selbständig und ohne fremde Hilfe von mir erstellt wurde. Es wurden nur die angegebenen Quellen und Hilfsmittel verwendet. Aus Publikationen und Werken entnommene Stellen wurden als solche kenntlich gemacht. Diese Arbeit wurde noch keiner anderen Prüfungsbehörde vorgelegt, auch nicht veröffentlicht.

Halle/Saale, 31.08.2006

Amir Abushouk

Publications:

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