1. Introduction

1.1 Epidemiologic aspects of impaired lipid metabolism

Mammals have evolved mechanisms to store energy during periods of plenty, which helps to guarantee survival during periods of drought and famine. Excess nutrient is stored as triglycerides (TGs), primarily in the adipose tissue, but in other tissues as well. In addition to the beneficial effects of nutrient storage, however, the long-term storage of excessive amounts of lipid can have a negative impact on health, especially under conditions of longer life span and decreased physical activity. As a consequence of sustained overnutrition, obesity has become epidemic in industrialized countries and is increasingly common in developing countries worldwide. The prevalence rates are continuing to rise and obesity is occurring in all groups at younger ages. Very worrisome are the concurrent and parallel increases in the prevalence of pathologic conditions associated with obesity, which include type 2 diabetes mellitus, CVD, hypertension, hypercholesterolemia, hypertriglyceridemia, nonalcoholic fatty liver disease, arthritis, asthma, and certain forms of cancer (Shoelson et al., 2007).

Obesity leads to impaired lipid metabolism, which is believed to be one of the main reasons for development of the above mentioned complications. While plasma LDL levels and rate of oxidation of LDL remain conventionally known risk factors for CVD, in the last decade increased the importance related to plasma TG levels, especially in postprandial phase, as an independent risk factor. TGs are transferred to body tissues within triglyceride-rich lipoproteins (TRLs), which include chilomicrons and VLDL. In the postprandial phase due to limited lipoprotein lipase (LPL) availability, competition at the level of LPL occurs, resulting in accumulation of TRLs. This competition is most likely when fasting hypertriglyceridaemia is present,
as occurs in the metabolic syndrome, type 2 diabetes and familial combined hyperlipidaemia. However, it has also been suggested that of all patients with premature CVD, 40% have normal fasting plasma lipids, whereas most of these patients have impaired clearance of postprandial lipoproteins (Alipour et al., 2007).

1.2 Atherosclerosis and lipid particles

Atherosclerosis is the number one reason of mortality in the Western world. The composition of the diet is believed to play a significant role in atherosclerosis development, with the amount and type of fat present being one of the most important factors. Dietary lipids, including fats and cholesterol, enter the blood in chylomicrons, large TRLs, which then undergo rapid lipolysis by LPL, resulting in the loss of some of the TGs and leaving smaller chylomicron remnants (CMRs), which are removed from the circulation by the liver (Botham et al., 2007). TRLs are mainly produced postprandially, and people in the Western world are non-fasting for most of the day, which leads to a continuous challenge of the endothelium by atherogenic lipoprotein remnants. Endogenous TRLs (VLDL) and exogenous TRLs (chylomicrons) share the same metabolic pathway, e.g. endothelium-bound LPL that hydrolyses TGs into glycerol and three fatty acids (Alipour et al., 2007).

1.3 The association between lipid metabolism, inflammation and atherosclerosis

Since the works of Hansson et al have been published (Jonasson et al., 1985), atherosclerosis is no longer considered to be a primary disorder of lipid accumulation. It is a disease of disordered immunity in which there is a dynamic interaction between endothelial dysfunction and inflammation (Mahmoudi et al., 2007; Robertson and Hansson, 2006). Many inflammatory markers have been associated with CVD, such
as C-reactive protein (CRP), leucocyte count and complement C3 (Halkes et al., 2001; van Oostrom et al., 2007; van Oostrom et al., 2003). Several studies with animal models showed reduced plaque formation (Eriksson et al., 2001; Huo and Ley, 2001) and prevention of endothelial dysfunction (Murohara et al., 1994) when adherence of leucocytes to the endothelium was prevented. However, the priority of initializing factors and interactions between lipid particles and inflammatory response has not been established yet. Van Oostrom et al. have shown that postprandially, when TG and glucose rise, neutrophil counts increase, with concomitant production of pro-inflammatory cytokines and oxidative stress, and that these changes may contribute to endothelial dysfunction (van Oostrom et al., 2003). Fasting leucocytes of patients with CVD have increased lipid content when compared with controls and it was suggested that this was due to the uptake of chylomicrons in the bloodstream (Tertov et al., 1992). Postprandial C3 increment after a fat meal has been shown in healthy subjects and in patients with CVD (Halkes et al., 2001; Meijssen et al., 2002). This postprandial increment has been related to TG and non-esterified fatty acids (NEFA) metabolism (Alipour et al., 2007; Halkes et al., 2001).

1.4 Epidemiology and risk factors influencing the development of atherosclerosis

Large number (about 300) of risk factors has been associated with atherosclerosis and its major complications, CVD and stroke. However, between 70% and 90% of the risk of atherosclerotic disease can be explained by different associations between conventional risk factors, such as smoking, abnormal lipids, hypertension, diabetes, obesity, psychosocial factors, unhealthy diet, and lack of physical activity (Vinereanu, 2006). The risk of atherosclerotic cardiovascular disease is increased 2- to 3-fold in type 2 diabetes mellitus (Stamler et al., 1993) and metabolic syndrome (Liang et al.,
In type 2 diabetes both insulin resistance and hyperglycemia may promote atherosclerosis and its complications, while insulin resistance is likely a key underlying factor driving the complications of metabolic syndrome including atherosclerosis (Liang et al., 2007).

1.5 Lipid particles and macrophages
Atherosclerosis is believed to be initiated when macrophages which invade the artery wall take up lipid from the plasma lipoproteins and become foam cells (Libby et al., 1996). A large number of studies showed that modification of LDL particles, either chemically or by oxidation, is necessary before extensive lipid accumulation is induced (Albertini et al., 2002). In contrast, several studies have demonstrated that chylomicron remnants (CMRs) cause macrophage foam cell formation without prior oxidation (Batt et al., 2004; Botham et al., 2005; Yu and Mamo, 2000). Because of the large size of chylomicrons, it has been assumed that they cannot enter the vessel wall nor interact with artery wall macrophages. However, it has been shown that the smaller chylomicron remnant particles may be taken up by several types of macrophages and cause extensive TG and cholesterol accumulation, thus inducing foam cell formation (Umeda et al., 1995). Current evidence indicates that chylomicron remnants, particularly when their removal from the serum is delayed, are an independent risk factor in the pathogenesis of atherosclerosis (Wilhelm and Cooper, 2003). Several mechanisms are considered to play a role in the uptake of chylomicron remnants by macrophages (Napolitano et al., 2001; Van Lenten et al., 2001), however, there is no agreement about these mechanisms of uptake. Thus, though it is now clear that chylomicron remnants are pro-atherogenic, events leading to their incorporation by macrophages are poorly understood. The most generally
accepted pathway for macrophage uptake of chylomicron remnants is via the LDL receptor mediated by the apoE (apolipoprotein E) component of chylomicron remnants functioning as the LDL receptor ligand (Koo et al., 1988). However, from studies in cells devoid of LDL receptor activity, there is evidence that there may be chylomicron remnant uptake pathways in macrophages independent of the LDL receptor, and that uptake may also be mediated by other mechanisms (Bravo and Napolitano, 2007; Fujioka et al., 1998). Because most studies have been undertaken on lipoprotein particles, the mechanism of uptake of TGs has been remained unknown. However, at least one study demonstrated that TGs from TG-rich emulsions without lipoprotein particles have been uptaken by macrophages in a coated pit-dependent mechanism and elevate the level of intracellular TGs (Carvalho et al., 2002). Although TGs are transported to tissues by TRL, these lipoprotein particles contain also apoproteins, cholesterol, lipid-soluble vitamins and other lipid molecules. In our study, we put a goal to isolate the lipotoxic effect of TG from the other lipid components influencing macrophages, from the step of TG uptake until the cell death.

1.6 Reactive oxygen species (ROS) in macrophages

ROS are mostly produced as byproducts of oxidative phosphorylation (OXPHOS) pathway involved in energy production in mitochondria. ROS have a wide range of potential actions that are influenced by the specific moiety generated, its localization, amount, and proximity to other radicals, enzymes, and signaling molecules (Cave et al., 2006). Conventionally, in phagocytic immune cells, ROS have been considered to function primarily in host defense as antimicrobial factors. However, strong evidence supports a role for ROS in the regulation of pivotal cellular signaling events involved
in homeostasis, cell proliferation and differentiation, and inflammatory and immune responses (Touyz, 2005; Wenger et al., 2005). In this regard, ROS fulfill important prerequisites for intracellular messenger molecules: they are small, diffusible, and ubiquitous molecules that can be rapidly synthesized and destroyed, thus conferring spatial and temporal specificity to signaling events (Tonks, 2005; Wolin et al., 2005). However, because of their toxicity, there is a narrow concentration range in which they can function effectively as second messengers (Fialkow et al., 2007). Transduction of the chemical ROS signal into a biologically relevant event is mediated by posttranslational covalent modification of specific amino acid residues on proteins, resulting in a change in protein function. In health, ROS generation is counteracted by the activity of enzymatic and nonenzymatic antioxidant systems that scavenge or reduce ROS levels, thereby maintaining an appropriate redox balance in cells and tissues. Perturbation of this normal balance due to increased ROS production and/or reduced antioxidant pool leads to a state of oxidative stress, namely an enhanced susceptibility of biological molecules and membranes to reaction with ROS (Cave et al., 2006).

In macrophages and neutrophils, oxidative burst is characterised by massive production of ROS in an inflammatory environment. In an inflammatory environment, activated neutrophils and macrophages produce large quantities of superoxide radical and other ROS via the phagocytic isoform of NAD(P)H oxidase (Keisari et al., 1983). There exists evidence that the intracellular redox state also modulates the immunological functions of macrophages (Valko et al., 2007).

The normal balance of ROS and ROS-neutralizing factors in cells is dependent of the redox state of compounds responsible for neutralizing effect. The redox state of a compound can be defined as the tendency to accept or donate electrons. As ROS are
potent oxidizing agents, they can affect the local or general cytosolic balance of oxidation/reduction ("redox state") (Hansen et al., 2006). Under physiological conditions, the cellular redox state is characterized by a reducing environment of the cytosol. The major redox “buffer” in the cytosol is glutathione, and the vast excess of reduced over oxidized glutathione is largely responsible for the reducing potential of the cytosol. In leukocytes, like other cell types, ROS and alterations in cellular redox state influence signaling pathways in diverse ways and, importantly, participate in the signaling cascade triggered by inflammatory mediators, including cytokines and chemoattractants (Fialkow et al., 2007).

1.7 Cell death

1.7.1 Apoptosis

In a pioneering study on ischemic liver injury, published in 1972, Kerr et al. (Kerr et al., 1972) observed a novel type of cell death, called "apoptosis," which appeared different from toxin-induced necrotic hepatocyte death. As revealed by electron microscopy, apoptotic cells form small round bodies that are surrounded by membranes and contain intact cytoplasmic organelles or parts of the nucleus. These bodies result from progressive cellular condensation and budding, and eventually are engulfed by resident phagocytic cells. The morphological changes that define apoptosis are chromatin condensation and nuclear fragmentation (Kroemer et al., 2005). Apoptosis can be viewed as a process that eliminates excessive, damaged, or mutated cells. Disabled apoptosis is a pathogenic event that contributes to oncogenesis and cancer progression. Unwarranted apoptosis of postmitotic cells also causes disease. Acute massive apoptosis participates in the pathophysiology of infectious diseases, septic shock, and intoxications (Reed, 2002). The best
characterized and the most prominent types of apoptosis are called the extrinsic and intrinsic pathways. In the extrinsic pathway (also known as "death receptor pathway"), apoptosis is triggered by the ligand-induced activation of death receptors at the cell surface. In the intrinsic pathway (also called "mitochondrial pathway"), apoptosis results from an intracellular cascade of events in which mitochondrial permeabilization plays a crucial role (Scaffidi et al., 1998). It appears that the activation of a specific class of proteases, the caspases ("cysteine protease cleaving after Asp"), is required for the rapid manifestation of these features of apoptosis. However, not all caspases are required for apoptosis and the process generally results from the activation of a limited subset of caspases, in particular, caspases-3, -6, and -7 (Fuentes-Prior and Salvesen, 2004). These are the "executioner" caspases, and they mediate their effects by the cleavage of specific substrates in the cell (Kroemer et al., 2007).

1.7.2 Necrosis (oncosis)

Necrosis may be viewed as an accidental type of cell death. Necrosis is not genetically predetermined and normally occurs within a short period following the triggering insult (2–4 h). The final phenotypic appearance of necrotic cells is highly dependent on the severity of the injury. The main features of necrosis include a gain in cell volume (oncosis) that finally leads to rupture of the plasma membrane and the unorganized dismantling of swollen organelles. Necrosis lacks a specific biochemical marker. Necrosis is considered to be harmful because it is often associated with pathological cell loss and because of the ability of necrotic cells to promote local inflammation that may support tumor growth (Kroemer et al., 2007; Vakkila and Lotze, 2004).
However, in practice, the definitions of apoptotic and necrotic cell death types are less clear. In addition to the primary oncotic process, a cell that is undergoing apoptosis may exhaust cellular adenosine triphosphate (ATP) levels and then progress to secondary necrosis characterized by cell swelling and lysis. On the other hand, if the oncotic cell death mechanism is inhibited, the stress may eventually force the cell into apoptosis (Jaeschke and Lemasters, 2003). One of the concepts connecting between apoptosis and necrosis defines that an insult affecting only a few mitochondria would be resolved by autophagy of the damaged cell organelles. However, if it involves more mitochondria, which release enough cytochrome $c$ to activate the caspase cascade, and if the remaining intact mitochondria maintain the cellular ATP levels, the cell will undergo apoptosis. On the other hand, if the insult is too severe, cellular ATP levels are not maintained and the cell will die by oncotic necrosis (Jaeschke et al., 2004).

1.7.3 Other types of cell death

1.7.3.1 Autophagic cell death

While apoptosis involves the explosive activation of catabolic enzymes leading to the demolition of cellular structures and organelles, autophagy is a slow, circumspect phenomenon in which parts of the cytoplasm are sequestered within double-membraned vacuoles and finally digested by lysosomal hydrolases (Kroemer and Jaattela, 2005). The functional relationship between apoptosis and autophagy is complex, and autophagy may either contribute to cell death (Shimizu et al., 2004) or constitute a cellular defense against acute stress, in particular induced by deprivation of nutrients or obligate growth factors (Baehrecke, 2005; Boya et al., 2005). Cells that are deprived from exogenous energy sources catabolize part of their cytoplasm to
generate ATP and other intermediate metabolites that allow them to meet their essential energetic demand. Autophagy is essential for the removal of damaged mitochondria. Moreover, autophagy allows for the turnover of cytoplasmic regions including protein aggregates and damaged organelles. Thus autophagy prevents the accumulation of misfolded proteins in inclusion bodies. However, it is complicated to define in which cases autophagic cell death truly occurs through autophagy, meaning that inhibition of autophagy would prevent cell death, and in which cases it occurs with autophagy meaning that inhibition of autophagy would only affect the morphology of the process, but not the fate of cells (Kroemer et al., 2007).

1.7.3.2 Mitotic catastrophe

Mitotic catastrophe represents a type of cell death that occurs during mitosis. Thus the morphological aspect of cells dying during mitosis is different from that of cells dying from classical apoptosis, which mostly occurs in the interphase. Mitotic catastrophe often involves micronucleation and multinucleation events that occur before cell death. Mitotic catastrophe results from a combination of deficient cell cycle checkpoints (in particular the DNA structure and the spindle assembly checkpoints) and cellular damage (Castedo et al., 2004). Failure to arrest the cell cycle before or at mitosis triggers an attempt of aberrant chromosome segregation, which culminates in the activation of the apoptotic pathway and ultimately leads to the cellular demise. Cell death occurring during the metaphase/anaphase transition is often characterized by the activation of caspase-2 (which can occur in response to DNA damage) (Castedo et al., 2004) and/or MMP with release of cell death effectors such as AIF and the caspase-9 and -3 activator Cyt c (Kroemer et al., 2007; Muchmore et al., 1996).
1.7.4 Role of macrophage cell death in formation and progression of atherosclerotic plaque

Dead macrophages are frequently observed in human atherosclerotic lesions, and are considered to be involved in atherosclerotic plaque instability (Feng et al., 2003). Indeed, unstable plaques demonstrate a greater portion of apoptotic cells than stable ones (Kolodgie et al., 2000; Moreno et al., 1994). Immunohistochemical staining of ruptured plaques has shown that apoptotic nuclei in plaque rupture sites are essentially those of macrophages and much less frequent of smooth muscle cells (SMC) and T lymphocytes (Kolodgie et al., 2000). Fibrous caps of ruptured plaques not only contain more macrophages, but also less SMC than those of unruptured ones. Death of macrophages and SMC in blood vessels is a complicated process owing to their mutual interactions. Thus, the presence of monocytes and macrophages in plaques also increases the rate of SMC apoptosis (Seshiah et al., 2002). In an advanced atherosclerotic plaque region, the apoptotic processes may lead to massive macrophage cell death, and an impaired capacity of other macrophages to clean and clear the plaque regions from cell debris may lead to the development of a necrotic core and accelerated inflammatory process in the intima. Prior to their death, macrophages express multiple metalloproteinases (e.g. stromelysin) and serine proteases (e.g. urokinase) that degrade the extracellular matrix, weakening the plaque and making it prone to rupture.

1.8 Mitochondria and mitochondrial biogenesis

1.8.1 Involvement of mitochondria in ROS generation and neutralization

In the mitochondrial electron transport chain, complex IV retains all partially reduced intermediates until full reduction of oxygen is achieved (Turrens, 2003). Other
complexes may leak electrons to oxygen, partially reducing this molecule to $O_2^{-}$. Complex I and III are the primary source of $O_2^{-}$ production in mitochondria (Chen et al., 2003; Nicholls and Budd, 2000). In the absence of ADP, electrons derived from succinate (FADH$_2$-linked complex II substrate) can reversely flow to complex I generating increased $O_2^{-}$ production, and, for this reason, complex I is considered the major physiologically and pathologically relevant ROS-generating site in mitochondria (Han et al., 2003; Liu et al., 2002). Superoxide anions in the mitochondrial matrix are quickly dismutated to hydrogen peroxide ($H_2O_2$). In mitochondria, $H_2O_2$ is reduced to water by the enzymes glutathione peroxidase or catalase (Chance et al., 1979; Madamanchi and Runge, 2007).

ROS produced initially in mitochondria or by enzyme sources such as NAD(P)H oxidase in the cell may act in a positive feedback, leading to more ROS production from mitochondria in a process termed ROS-induced ROS release (Brandes, 2005). In addition to being a major site of ROS production, mitochondria are compromised by severe and/or prolonged oxidative stress. Oxidative modifications of mitochondrial proteins, lipids, and mtDNA result in loss of function (Di Lisa and Bernardi, 2005).

1.8.2 Assembly of mitochondrial subunits

All the subunits of complex II are encoded by nuclear genes, whereas the subunits of the other complexes are encoded by both nuclear and mtDNA. Most cells contain hundreds of mitochondria, and each mitochondrion contains 5 to 10 copies of mtDNA (Wallace, 1999). The mtDNA contains 13 genes coding for polypeptides essential for OXPHOS, 12S and 16S ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes required for protein synthesis in mitochondria (Madamanchi and Runge, 2007).
Nuclear-encoded subunits of mitochondrial complexes are assembled into the inner mitochondrial membrane by a mechanism which demands a complicated coordination of protein activity. Recent studies of subunits of cytochrome oxidase (complex IV) shed light on this biological assembly process revealing an astonishingly complex procedure by which the different subunits of the enzymes are put together and the required cofactors are supplied. According to this model, following their synthesis and integration into the inner membrane, Cox1 and Cox2 are individually modified and equipped with their respective cofactors in several reactions. These reactions appear to occur in a sequentially ordered manner forming two parallel assembly lines which finally merge to form a core complex. Substrate-specific chaperones bind to Cox1 and Cox2, thereby maintaining these proteins in an assembly-competent state. Upon association of the residual nuclear encoded subunits with the core complex, the final holoenzyme is formed and the assembly reaction completed. (Herrmann and Funes, 2005). This process emphasizes the complication of regulation of mitochondrial biogenesis.

1.8.3 Regulation of mitochondrial biogenesis

Recent studies establish associations between metabolic syndrome and mitochondrial dysfunction. One likely explanation for the mitochondrial dysfunction observed in the prediabetic state is a reduced expression of genes involved in mitochondrial oxidative metabolism, as has been observed in type 2 diabetes (Mootha et al., 2003; Patti et al., 2003). Many of the oxidative genes are under transcriptional control of PPARγ coactivator-1α (PGC-1α), which is an inducible coregulator of nuclear receptors such as nuclear respiratory factor 1 (NRF1) involved in the control of mitochondrial biogenesis and functions (Handschin and Spiegelman, 2006; Kelly and Scarpulla,
The PGC-1α, initially identified as a PPARγ interacting protein in brown adipose tissue (Puigserver et al., 1998), is the founding member of a family of three related proteins that control major metabolic functions through the coactivation of nuclear receptors and other transcription factors (Finck and Kelly, 2006; Lin et al., 2005). PGC-1 family members cooperate to control mitochondrial functions such as oxidative phosphorylation and mitochondrial biogenesis (Feige and Auwerx, 2007; Scarpulla, 2006).

In addition to the down-regulation of the OXPHOS gene set, it has been shown that the expression of PGC-1α itself is also reduced in patients with type 2 diabetes (Mootha et al., 2003; Patti et al., 2003; Schrauwen, 2007). In muscle and adipose tissue, prediabetic and diabetic state is characterized by a decreased expression of genes involved in oxidative phosphorylation, many of which are regulated by NRF-dependent transcription (Patti et al., 2003). These data indicate that decreased PGC-1α expression may be responsible for the reduced expression of metabolic and mitochondrial genes regulated by NRF and may contribute to the metabolic disturbances characteristic of insulin resistance, diabetes mellitus, and obesity (Nisoli et al., 2007). Although a possible role of PGC-1α in atherogenesis has not been addressed in animal models, studies in human, bovine, and mouse endothelial cells suggested that overexpression of PGC-1α reduces ROS accumulation and apoptotic cell death under basal and oxidative stress conditions (Igléseder et al., 2006; Valle et al., 2005).
1.9 Hypothesis and aims

1.9.1 Hypothesis

It is well-known that high levels of free fatty acids are toxic to cells, however, the question about the toxicity of TGs remains open. We hypothesize that TG uptake by macrophages directly affects cellular redox state through impairment of mitochondrial activity and leads to necrotic cellular death.

1.9.2 Aims

General:

To elucidate the molecular mechanisms underlying TG-induced lipotoxicity in macrophages.

Specific:

- To study the effect of triacylglycerol (TG) uptake on macrophage cell death.
- To elucidate the mechanism by which TG regulate oxidative stress and ROS production and the role of antioxidants in this pathway.
- To investigate the effect of TG on genes involved in mitochondrial biogenesis and mitochondrial function.
2. Materials and methods

2.1 Materials

ABgene, England:

Reverse-iT 1\textsuperscript{st} strand synthesis kit.

Alltech, Israel:

MetPREP.

Amersham pharmacia biotech, England:

Acrylamide solution, TEMED, nitrocellulose membrane, ECL, dithiotreitol (DTT).

Applied Biosystems:

SYBR Green Master mix.

BDH, England:

dimethylsulfoxide (DMSO).

Bio Lab LTD, England:

Bradford reagent- Bio Rad protein assay.

Biological industries, Beit Ha Emek, ISRAEL:

RPMI 1640 medium,

Penicillin-Streptomycin 10000 units/ml,

Glutamine.

Braun, Germany:

Lipofundin, soybean oil TG-based lipid emulsion.

Boehringer Biochemical Mannheim, Germany:

30\% Acrylamid-Bis acryl amid, Proteinase inhibitor cocktail tablets.

Calbiochem, Germany:

Caspase-3 substrate II, fluorogenic.
Chem Service USA:
Paraoxon.

Frutarom Chemicals LTD, Haifa, Israel:
KCl, NaOH, KOH, NaCl, Methanol.

Gibco, USA:
Agarose, DMEM medium, FCS, penicillin, streptomycin, glutamine.

JT Baker, USA:
Acetonitril, Tween-20.

Lyco Red, Israel:
Lycopene.

Merck, Germany:
Glycerol, KH$_2$PO$_4$, NaCl.

Molecular probes, USA:
H$_2$DCF-DA.

Promega, USA:
Nuclease free water.

Sigma, Israel:
Ammonium persulfate, ascorbic acid, Bradford reagent, Bromophenol Blue, Bovine serum albumine (BSA), butathione sulfoxime (BSF), C17 fatty acid, chlorophorm, ethidium bromide, glutathione, glycine, isopropanol, KH$_2$PO$_4$, lipoic acid, beta-mercaptoethanol, N- acetyl cysteine (NAC), NADH, Na-Selenite, Nile red, propidium iodide, phosphoric acid, quercetin, resveratrol, rotenone, SDS, skim milk powder, Tris, tritonX100, tri- reagent, Tween 20, TEMED, toopherol, Trypan Blue.
2.2 Methods

2.2.1 Cell culture
Murine J774.2 macrophages were cultured in RPMI medium enriched with 10% fetal calf serum, 1% glutamine, and 1% penicillin-streptomycin. Cells were maintained in an incubator with temperature (37°C) and CO2 (5%) control. Prior to experimental procedures, macrophages were seeded on 6-well plates at a concentration of 100,000 cells/ml.

2.2.2 Lipid treatments
J774.2 macrophages were exposed to several lipid sources: commercial lipid emulsions based on soybean or olive oil TGs (final concentration 1 mg/ml) or BSA-complexed palmitic acid (150 µM), linoleic acid (600 µM) or oleic acid (600 µM). Free fatty acids (FFA) were dissolved in ethanol at a concentration of 100 mM. Then BSA water solution (20%, w/v) was heated to 45°C, and the FFA solution was gradually added to the BSA to achieve a complexed FA stock solution (8 mM) (Svedberg et al., 1990; Thompson et al., 2000).

The composition of soybean and olive oil-based lipid emulsions are demonstrated in Table 1 and total composition of both emulsions – in Table 2.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>ClinOleic 20% (olive oil-based)</th>
<th>Lipofundin MCT/LCT 10% (soybean oil-based)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT</td>
<td>0</td>
<td>50%</td>
</tr>
<tr>
<td>C16:0</td>
<td>13.5%</td>
<td>5.0%</td>
</tr>
<tr>
<td>C18:0</td>
<td>2.9%</td>
<td>2.0%</td>
</tr>
<tr>
<td>C18:1</td>
<td>59.5%</td>
<td>12.0%</td>
</tr>
<tr>
<td>C18:2 (n-6)</td>
<td>18.5%</td>
<td>27.0%</td>
</tr>
<tr>
<td>C18:3 (n-3)</td>
<td>2.0%</td>
<td>4.0%</td>
</tr>
</tbody>
</table>

Table 1
Composition of fatty acids (% of total fatty acids) in soybean and olive oil-based lipid emulsions.
Total composition of 1000ml of commercial soybean and olive oil-based lipid emulsions.

<table>
<thead>
<tr>
<th>Components in 1000ml</th>
<th>ClinOleic 20% (olive oil-based)</th>
<th>Lipofundin MCT/LCT 10% (soybean oil-based)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refined oil (gr)</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>MCT (gr)</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Egg phosphatides/lecithin (gr)</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Glycerol (gr)</td>
<td>22.5</td>
<td>25</td>
</tr>
<tr>
<td>Sodium olate (gr)</td>
<td>0.3</td>
<td>QNM*</td>
</tr>
<tr>
<td>Sodium hydroxide for pH</td>
<td>QNM*</td>
<td>NM**</td>
</tr>
<tr>
<td>Alpha-Tocopherol</td>
<td>NM**</td>
<td>QNM*</td>
</tr>
</tbody>
</table>

*QNM- quantity not mentioned; **NM- not mentioned in composition list.

2.2.3 Analysis of spontaneous hydrolysis of LE

Before treating the cells with a soybean oil TG-based lipid emulsion, the hydrolysis level of the FFA was evaluated by thin-layer chromatography (TLC). The LE was analyzed relative to TG and FFA standards on a silica gel plate. The running solvent contained petrol ether, diethyl ether, and acetic acid in a volumetric ratio of 80:20:1. The retention factor (Rf) of the compounds on the plates was then visualized with iodine.

2.2.4 Hydrolysis of lipid emulsion and release of free fatty acids

Soybean-based LE was hydrolyzed by KOH (10M). The hydrolysis was undertaken for 3h at 80°C. The hydrolizate was neutralized to pH 7.0, with HCl, and ethanol was added to the final concentration of 20% (v/v) to dissolve the FA salts produced. The efficiency of the hydrolysis was determined using TLC chromatography tested by running the products on TLC, as described above.

2.2.5 Analysis of peroxide value of LE

The peroxide value (PV) of the commercial emulsion was measured using the American Oil Chemists' Society's method. Briefly, the lipids were extracted from the LE by chloroformic extraction (1:1:2 LE: chloroform: methanol). Extracted oil (5 mg)
was dissolved in a 30-ml acetic acid:chloroform (3:2) mixture and 0.5 ml of saturated potassium iodide solution and 30 ml of distilled water were added. The mixture was titrated with 0.01 M sodium thiosulfate until its yellow color almost disappeared. Then 0.5 ml of a 1% (w/v) starch solution was added to the mixture, and the titration continued until all of the color disappeared. The PV was calculated using the equation:

\[
PV \text{ (milliequivalents peroxide per kilogram sample)} = S \times N \times 1000 / g \text{ sample},
\]

where \( S = ml \text{ Na}_2\text{S}_2\text{O}_3 \) and \( N = \text{normality of the Na}_2\text{S}_2\text{O}_3 \text{ solution} \) (JAOAC, 1949).

LE was added to the cell culture at a concentration of 0.1% (w/v) TG (1 mg lipids/ml) (Vazquez et al., 1994). Physiological range of TG in plasma is up to 1.5 mg/ml. The cells were incubated with the LE for 12, 24, or 48 h, and then washed twice with phosphate-buffered saline (PBS), and intracellular ROS and cell viability were measured. Other agents, such as antioxidants or cycloheximide (CH), were added as specified in the relevant figure legends.

2.2.6 Detection of cellular fatty acid profile with high-resolution gas chromatography analysis

The cellular concentrations of fatty acids were measured using gas chromatography (GC). The cells in medium were centrifuged (600xg, 5 min) and resuspended in PBS. Protein concentration was determined by the Bradford method, cells were centrifuged again at 600g and the supernatant was removed. A mixture of chloroform: methanol (2:1) was added to the cells, and samples were mixed and kept at room temperature for 30 min. Distilled water (20%, v/v) was then added and the phases separated. The chloroform phase was dried under nitrogen. An internal standard of C17-fatty acid
was added and the dried samples were resuspended in toluene and treated with a hydrolysis-methylation reagent (MetPREP), according to the manufacturer's directions. Analysis was performed with an HP-4890 (Agilent Technologies) GC equipped with a flame ionization detector (FID) and DB-WAX capillary column (60 m × 0.32 mm i.d., df = 0.25 µm) (J and W Scientific, Folsom, CA). The samples were injected at an injector temperature of 260°C and split 1/80 for 1 min. Operating conditions were as follows: the column was held at 180°C for 2 min and then increased by 10°C/min to 230°C where it was held for 20 min. Helium was used as the carrier gas with a linear velocity of 31 cm/s. Detector temperature was 250°C (Bezman et al., 2003). Quantification of the results was based on the area of the internal standard peak.

2.2.7 Evaluation of intracellular lipids

After exposure of the J774.2 macrophages to different lipid treatments, the cells were collected, washed twice and resuspended in PBS. Following this procedure, the cells were stained with DMSO-dissolved Nile Red in a concentration of 1 µg/ml. Flow cytometry measurement was carried out using FL2 channel (excitation at 488 nm and emission at 575 nm). Data were collected from 10,000 cells.

2.2.8 Cell viability

Cell membrane integrity was detected as previously described (Tirosh et al., 2001). Briefly, cells were stained with 2 µg/ml propidium iodide (PI) and measured by flow cytometry, at the following fluorescence setting: excitation at 488 nm and emission at 575 nm. Data were collected from 10,000 cells.

2.2.9 DNA integrity and cell cycle
Cells exposed to LE were centrifuged (600xg, 5 min). The pellet was resuspended in 1% (w/v) paraformaldehyde, incubated for 30 min, and centrifuged. The resultant pellet was resuspended in a solution containing 50 µg/ml PI, 0.1% (w/v) sodium citrate, and 0.1% (v/v) Triton X-100. The permeabilized cells were kept in the dark at 4°C for 1h. DNA integrity was analyzed by FACS using a log scale and measurement of cell cycle using a linear scale, FL2 channel, excitation at 488 nm and emission at 575 nm. Data were collected from 10,000 cells (Aronis et al., 2003).

2.2.10 ROS measurements

Intracellular ROS were detected using a H2DCF-DA fluorescent probe (Tirosh et al., 2000). The cells were incubated with 25 µM H2DCF-DA for 30 min at room temperature. The fluorescence was measured in a FACS, with excitation at 488 nm and emission at 530 nm. Data were collected from 10,000 cells. Superoxide was measured by the use of nitro blue tetrazolium (NBT). After 12, 24, and 48 h exposure to LE, the medium was removed, and fresh medium containing 0.1% (w/v) NBT was added to the cells. Incubation took place in an incubator with controlled humidity (37°C, 5% CO2) for 15 min. The cells were then centrifuged (600g, 5 min), the supernatant was removed, and the pellet was treated with 1 ml dimethyl sulfoxide (DMSO) to extract the formazan. After centrifugation, absorbance of the supernatant was measured by spectrophotometer at 520 nm. The results were adjusted to milligram protein (Baehner and Nathan, 1968).

2.2.11 Annexin V-PI double staining

Cells were washed twice in phosphate-free binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM CaCl2, pH 7.4) and centrifuged, and the supernatant was removed. Annexin V was dissolved in the same buffer (0.2 µg/ml) and added to the cells. After
a 30-min incubation at room temperature in the dark, 0.2 µg/ml PI was added, and measurements were made by FACS, at settings FL1 (excitation 488 nm, emission 530 nm) and FL3 (excitation 488 nm, emission 675 nm).

2.2.12 Determination of caspase-3 (DEVDase) activity

Necrotic cell death is a major pathway in the absence of caspase activation. Classical apoptotic stimuli such as Fas activation are reported to facilitate the necrotic pathways in cells that do not express caspase-8 and do not activate downstream caspases in response to oligomerization of Fas-associated protein with death domain (FADD) (Holler et al., 2000; Matsumura et al., 2000). For analysis of caspase activity, cells were incubated for 2 h in ice-cold PBS containing 0.5% Triton X-100 and 5 mM dithiotreitol (DTT). The suspension of permeabilized cells was agitated slightly and centrifuged at 14,000g. The clear supernatant was tested for caspase-3 activity as previously described (Sen et al., 1999), using the caspase-3 substrate Ac-DEVD-AMC (Calbiochem, Darmstadt, Germany). Incubation was conducted in 200 µl of reaction mixture containing approximately 1 mg sample protein, in the dark, at 30°C for 4 h (linear phase of the reaction). Caspase-3 activity was expressed as arbitrary fluorescence units (AU) per mg protein.

2.2.13 Measurement of glutathione

Reduced glutathione (GSH) was measured by high-pressure liquid chromatography (HPLC). The cells and GSH standard were dissolved in 4% (v/v) metaphosphoric acid and run in running buffer (50 mM KH₂PO₄ and 2% v/v acetonitrile, pH 2.7) using a Synergy 4 μ Polar-RP 80A column (Phenomenex, Torrance, CA) when the cell potential was 800 mV. Detection was made by electrochemical detector. The results were reported as nanomole per milligram protein (Sen et al., 1999).
2.2.14 SDS-PAGE analysis of mitochondrial complexes

Total protein extracts from cells were obtained by lysing J774.2 and MPM cells in a buffer (10 mM Tris-HCl, 1% SDS, 1 mM sodium ortho-vanadate pH 7.4). Total protein (40 µg) was separated on a 12.5% SDS-polyacrylamide gel.

**Resolving gel:**
- Acrilamid solution – 4.2ml
- Resolving gel buffer (Tris-HCl 1.5 M, pH 8.8) – 2.5ml
- 10% SDS – 100µl
- ddH2O – 3.2ml
- Ammonium persulfate – 100µl
- TEMED – 4µl

**Stacking gel:**
- Acrilamid solution – 0.44 ml
- Stacking gel buffer (Tris-HCl 0.5 M, pH 6.8) – 0.83 ml
- 10% SDS – 33 µl
- ddH2O- 2 ml
- Ammonium persulfate –30 µl
- TEMED – 3µl

Separated proteins were transferred to nitrocellulose membranes using semi-dry transfer method following conventional protocols. Membranes were blocked in 1% of nonfat milk in TBST buffer (10 mM Tris, 150 mM NaCl, 0.1% Tween20 pH 7.5) for 2 h at room temperature. Then mouse monoclonal anti-NDUFS3, mouse monoclonal anti-NDUFA9, chicken polyclonal anti-NDUFA10, mouse monoclonal anti-
UQCRC2, or mouse monoclonal anti-CoxIV (all mentioned antibodies were purchased from Abcam Ltd, Cambridge, UK) were incubated overnight at 4°C. Primary antibodies were washed several times with TBST, and secondary horseradish peroxidase-conjugated antibodies (goat anti-mouse and rabbit anti-chicken) were used for 1 h at room temperature. Protein bands were visualized following 1 min incubation in enhanced chemiluminescence (ECL) detection reagents (Santa-Cruz Biotechnology) and exposure to high-performance chemiluminescence film (Fujifilm, Tokyo, Japan). All primary antibodies were diluted 1:2000. Secondary antibodies were applied in 1:4000 dilutions.

Following the protein visualization, the membranes were stripped with stripping buffer:

Tris – 162.5 mM
SDS – 2% (w/v)
beta-mercaptopethanol – 100mM
ddH₂O – 500ml

The membranes were placed in the stripping buffer for 30 min (50°C), then rinsed with TBST and exposed to mouse anti-beta actin (BD transduction laboratories, Pharmigen, CA) overnight. The anti-actin antibody was washed with TBST, and the membranes were exposed to horseradish peroxidase-conjugated anti-mouse secondary Ab for 1 hour and then visualized. All protein bands were related to beta-actin.

2.2.15 Isolation of RNA and Reverse Transcription reaction
Total RNA was isolated by separation using Tri-Reagent (0.5 ml/sample) and chloroform (0.1 ml/sample). The samples were incubated on ice for 10 min and subsequently centrifuged at 14,000 rpm for 10 min, 4°C. The upper layer was taken for further wash in isopropanol (0.25 ml/sample) in room temperature, and centrifuged at 30,000xg for 10 min. The pellet was subsequently washed with 75% ethanol (0.5 ml/sample) and centrifuged at 30,000xg for 10 min, 4°C. The RNA pellet was air-dried for 20 min and then dissolved in 40 µl of nuclease-free water. The concentration of total RNA was determined by UV light absorption at 260 nm in spectrophotometer (UV-1700, Shimadzu, Japan). The 260/280nm absorption ratio taken for the Reverse Transcription reaction was 1.8-2.2.

Reverse transcription reaction was carried out using Promega M-MLV reverse transcriptase kit according to manufacturer instructions. Briefly, 1 µg of RNA was taken for the reaction and incubated with random hexamers (0.5 µg/sample) in total volume of 15 µl in 70°C for 5 min. The samples were immediately transferred to ice to avoid the rearrangement of secondary structures. M-MLV reverse transcriptase (25units/sample), dNTP (10mM each) and reaction buffer were then added to the RNA, and RT reaction was carried out in PCR incubator (FTGENE2-D, Techne, England) at 42°C.

2.2.16 Real- time Polymerase Chain reaction

Real-time PCR was performed for quantification of mRNA expression using the SYBR Green PCR mix in an ABI Prism 7300 Sequence Detector System (Applied Biosystems). Total RNA (1 µg) was reverse-transcribed to cDNA using random hexamers. The following cycling conditions were used in PCR: 40 cycles at 95°C for 30 s, 60°C for 30 s and extension at 72°C for 30 s. The beta-actin mRNA was used as
an endogenous control. Melt-curve analysis showed a single peak for the primer sets of genes, indicating no primer dimer formation. RNA samples were used as non-RT controls to exclude interference by genomic DNA contamination. Slopes were (-3.0)-(-3.4) for all target and endogenous control genes. DNA dilutions for the samples were set such that ∆Ct between the target and the endogenous control genes was no more than five cycles.

Primers were purchased from Hy Laboratories Ltd (Israel) and are summarized in the table below:

<table>
<thead>
<tr>
<th>Gene (mouse)</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>beta - actin</td>
<td>Left: 5'- cta agg cca acc gtg aaa ag -3'</td>
</tr>
<tr>
<td></td>
<td>Right: 5'- ggg gtg ttg aag gtc tca aa-3'</td>
</tr>
<tr>
<td>PGC-1 alpha</td>
<td>Left: 5'- caa acc ctg cca tgg tta ag -3'</td>
</tr>
<tr>
<td></td>
<td>Right: 5'- tga caa atg ctc ttc gct tt -3'</td>
</tr>
<tr>
<td>PGC-1 beta</td>
<td>Left: 5'- aag aag cgc ttt gag gtg tt -3'</td>
</tr>
<tr>
<td></td>
<td>Right: 5'- tga aca cgc gaa ggt gat aaa -3'</td>
</tr>
<tr>
<td>NRF-1</td>
<td>Left: 5'- gtt ggt aca ggg gca aca gt-3'</td>
</tr>
<tr>
<td></td>
<td>Right: 5'- cca tca gcc aca gca gag ta -3'</td>
</tr>
</tbody>
</table>

### 2.2.17 Statistical analysis

Data were analyzed by t-test when two groups were compared or by one way ANOVA if more than two groups were analyzed, using SPSS statistical program. Differences were considered significant at probability levels of P < 0.05. The groups were compared using Fisher's exact test.
3. Results

3.1 Aim 1: To study the effect of triacylglycerol (TG) uptake on macrophage cell death.

3.1.1 Intracellular changes in lipid content

To examine whether TGs from the LE are taken up by the cells, J774.2 macrophages were exposed to soybean oil-based lipid emulsion for 12, 24 and 48h. Accumulation of TGs in the cells was measured using Nile Red fluorescent probe by flow cytometry. The Nile Red measurement demonstrated time-dependent accumulation of lipid droplets in macrophages following TG treatment (Fig. 1A). Cellular composition of fatty acids following exposure to LE was measured at 12, 24 and 48h after TG treatment and in untreated cells (Fig. 1B). Cellular levels of 18:1 and 18:2 fatty acids increased over time. This correlates well with the LE composition (Table 1, "Materials and Methods"), as soybean is rich in oleic and linoleic acids. This finding indicates that unsaturated fatty acids from the LE were taken up by cells.

TLC analysis of soybean LE using TG and FFA standards was undertaken on the cells and the medium. The analysis revealed an undetectable amount, if any, of FFA in the LE (Fig. 1C).
Fig. 1. **Effect of soybean TG treatment on intracellular TG and FFA accumulation in J774.2 macrophages.** (A) Accumulation of TGs in J774.2 cells following their exposure to soybean LE. J774.2 macrophages were exposed to LE for 12, 24 and 48h. After the exposure, the cells were stained with Nile Red dye, and the fluorescence was measured by flow cytometry. Representative FACS histogram is demonstrated on the upper panel of the A bar chart. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the previous time exposure, $P<0.05$. (B) J774.2 macrophages were incubated with 1 mg/ml soybean TG for 12, 24 and 48h, then hydrolyzed, and the fatty-acid profile was determined by gas chromatography. Bars represent averages ± SD (n=3). *, indicates significant increase from the untreated control group; † - significant increase from the previous time exposure, §- significant reduction in FFA content, $P<0.05$. (C, left) Lipids were extracted from LE and subjected to TLC. The retention factor ($R_f$) was compared to free fatty acid (FFA) and triacylglycerol (TG) standards. No significant amount of FFA was seen in the emulsion. (C, right) Analysis of decomposition of TG to FFA in the cell culture medium; cell culture medium (1), cell culture medium collected from cells after 48 h (2), cell culture medium supplemented with LE collected from cells after 12 h (3), same as 3 but collected after 24 h (4), same as 3 but collected after 48 h (5).
In addition to *in vitro* ability of macrophages to accumulate TGs, *in vivo* TG uptake by mouse peritoneal macrophages (MPM) was evaluated. Soybean LE was intraperitoneally injected to mice with previous thioglycollate-stimulated recruitment of MPM. Cells isolated 24h after LE injection, demonstrated higher levels of intracellular TG accumulation (Fig. 2, right panel) than cells from PBS-injected animals (left panel).

Fig. 2. Effect of intraperitoneal soybean lipid emulsion (LE) injection on intracellular TG accumulation in mouse peritoneal macrophages (MPM). Thioglycollate (4%) was injected IP to C57/Bl mice for 72h to induce local inflammation. 24h before the animals were sacrificed (48h after the thioglycollate injection), 2 ml of soybean LE had been injected IP to the treatment group and 2 ml of PBS to the control group. MPM were isolated and seed as described in "Materials and methods" section. The cells underwent Nile Red staining and imaging by fluorescent microscopy (x10).
3.1.2 Intracellular TG degradation and effect of lipase inhibitor on TG-treated cells

An ability of the cells to decompose and/or remove accumulated TGs was verified by Nile Red staining (Fig. 3A). The cells were preloaded with TGs for 12h, and then the TG-containing medium was replaced by non-TG supplemented medium. Measurements of cellular TG content were undertaken at the loaded stage and every 3h after the TG removal. The result demonstrated a linear time-dependent reduction in intracellular TG content after stopping their exposure to extracellular TGs.

An effect of broad spectrum lipase inhibitor, paraoxon (PO), on degradation of lipids in TG-treated cells was also examined (Fig. 3B). The cells were loaded with TGs for 12h with or without PO. Following this exposure, intracellular TG content increased in the presence of PO, demonstrating that TG uptake in macrophages is by most a lipase-independent process. Moreover, PO caused an additional statistically significant elevation of intracellular TG content, probably by decreasing TG degradation by intracellular lipases. After washing the TG from the medium, the macrophages were incubated with non-TG supplemented medium again with and without PO. PO-treated cells did not demonstrate TG degradation unlike non-PO treated cells that were able to decompose the preloaded TG (Fig. 3B).
Fig. 3. TG degradation ability of J774.2 macrophages: degradation kinetics with or without paraoxon (PO). (A) The cells were loaded with soybean oil TG (1 mg/ml) for 12h. Then the medium was replaced by non-TG supplemented medium and additional 12h incubation was undertaken. TG content was measured by Nile Red staining as described in "Materials and methods". Each point on the curve represents average ± SD (n=3). (B) The cells were loaded with soybean oil TG (1 mg/ml) with or without PO (20 µM) for 12h. Then the medium was replaced with non-TG supplemented medium with or without PO and an additional 12h exposure was undertaken. The resulting TG content in the macrophages was evaluated by Nile Red staining. The bars represent TG content in untreated (control) cells, TG-loaded cells and TG-loaded and then incubated with non TG supplemented medium. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the same time exposure without PO, §- significant difference from the same treatment in 12h TG-loaded cells, P<0.05.

3.1.3 Effect of TG on J774.2 macrophage viability

A dose-dependent increase in cell death was observed in macrophages treated with elevated concentrations of TGs (Fig. 4A), indicating their lipotoxic effect. Measurement of cell viability revealed that in 24 h, TG treatment led to a small, but significant increase in viable cells, however, longer exposure, for 48 h, resulted in cell death at the rate of 50% (Fig. 4B). Lipase inhibitor PO did not prevent and even enhanced cellular death when incubated with TG-treated cells (Fig. 4C). Taken together, the results of PO treatment (Fig 3B and 4C) demonstrate that uptake and
accumulation of TGs induce cell death by themselves and not due to their degradation to FFA.

Fig.4. Effect of soybean TG on viability of J774.2 macrophages. (A) J774.2 macrophages were incubated with soybean TGs (0.1 mg/ml, 1 mg/ml and 1.5 mg/ml) for 48 h. Cell viability was then measured, as described in "Materials and methods". Each point on the curve represents an average ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the previous dose exposure, \( P<0.05 \). (B) Cell viability measured after 12, 24 and 48h of treatment with TGs. Bars represent averages ± SD (n=3). *, indicates significant reduction from the untreated control group; § - significant increase as compared to untreated cells, \( P<0.05 \). (C) Cells were exposed to TG (1 mg/ml) and PO (20 µM) for 48h. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the same exposure without PO, \( P<0.05 \).
To characterize the type of the cell death, caspase-3 activity and presence of phosphatidyl serine flip-flop were evaluated. Treatment with TGs did not activate caspase-3 activity, as measured with DEVDase (Fig. 5A). Surprisingly, basal caspase-3 activity was suppressed by exposure to the LE. These data indicate that for the first 24 h, the natural apoptotic pathway was suppressed in the macrophages. Cellular staining with Annexin V and PI indicated activation of the necrotic pathway (Fig. 5B). TG treatment resulted in a loss of membrane integrity to both PI and Annexin V (upper right quadrant of the plot), indicating necrotic cell death (Fig. 5B).

![Fig.5](image.png)

**Fig.5. Effect of TG on cell death features of J774.2 macrophages: caspase-3 activity and flip-flop of membrane phosphatidyl serine.** (A) Cells were incubated with LE for 12, 24 and 48h. Caspase-3 activity as measured by DEVDase activity. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the previous time exposure, P<0.05. (B) Cells were incubated with TGs for 48h, and then necrosis vs. apoptosis assay was performed: dual staining of cells with propidium iodide (PI) and Annexin V. Fluorescence was measured by flow cytometry as described in "Materials and Methods". Upper plot represents the control cells and lower plot cells treated with LE for 48 h. In every plot: lower left quadrant represents viable cells, upper left- partial loss of membrane integrity (uptake of PI but not Annexin V), lower right- apoptotic cells and upper right- necrotic cells. Each quadrant carries information about the percentage of cells framed in it from an average of three experiments ± SD; *, indicates significant statistical difference from the upper plot, P < 0.05.
3.1.4 Lipotoxicity of olive oil TG in J774.2 macrophages

Accumulation of intracellular TGs was evaluated in J774.2 macrophages after their 12, 24 and 48h exposure to olive oil-based LE. Similar to soybean oil TG, olive oil-based TG led to time-dependent TG accumulation in the cells (Fig.6A), and reduced cellular viability at 48h (Fig.6B).

![Graph A](image1)

![Graph B](image2)

Fig.6. Accumulation of TGs and viability in J774.2 cells following their exposure to olive oil lipid emulsion. J774.2 macrophages were exposed to olive oil-based TG for 12, 24 and 48h. (A) After the exposure, the cells were stained with Nile Red dye, and the fluorescence was measured by flow cytometry. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the previous time exposure, \( P<0.05 \). (B) Cell viability measured by exclusion of PI (membrane integrity). Viability was measured as described in "Materials and methods". Bars represent averages ± SD (n=3). *, indicates significant reduction from the untreated control group; § - significant statistical increase compared to untreated cells, \( P<0.05 \).

Evaluation of TG-induced changes in cell cycle showed \( G_1 \) arrest (Fig. 7). \( G_1 \) phase arrest was observed following exposure to both soybean and olive TGs for 24h.
Reduction in the percent of cells at G$_2$ phase was observed at 24h, indicating that TGs composing soybean (Fig. 7B) and olive (Fig. 7C) oils affected the cell cycle in a similar way.

![Histograms showing cell cycle distribution](image)

Fig. 7. Changes in J774.2 macrophage cell cycle as a result of treatment with soybean or olive oil-based LE (1 mg/ml). Measurements were carried out as described in "Materials and methods". (A) Cell cycle of untreated cells. (B) Changes in cell cycle resulting from exposure to soybean oil TG (1 mg/ml) for 24h. (C) Changes in cell cycle resulting from exposure to olive oil TG (1 mg/ml) for 24h. Representative histograms are accompanied with statistical analysis (n=3). *, indicates a significant statistical difference from the untreated control cells, $P < 0.05$.

### 3.1.5 Lipotoxicity of FFA in J774.2 macrophages

The effect of the FAs which compose TGs in soybean-based LE on J774.2 macrophages was investigated by complete hydrolysis of the LE. The same concentration of hydrolyzed emulsion FA as TGs (1.1 mM) was then added to the cells. The FA of hydrolyzed TGs led to total cell death within 4h (Fig. 8), probably due to direct membrane damaging, indicating that this concentration was too high for investigating lipotoxic effects of FA.
Fig. 8. Cytotoxic effect of soybean TG-composing FA in J774.2 macrophages. Soybean TG was completely hydrolysed, and the cells were exposed to the fatty acids (1.1 mM) of the hydrolyzed emulsion. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the previous time exposure, \( P<0.05 \).

The minimal toxic concentration of palmitic acid in J774.2 macrophages was found to be 150 \( \mu M \). Exposure of the cells to 150 \( \mu M \) of PA caused cell death within 24h (Fig. 9A). In contrast to palmitic acid, OA and LA were not toxic at 150 \( \mu M \); exposure of macrophages to higher concentrations of these FAs (300-600 \( \mu M \)) for an extended period of time (48 h) was required to induce cell death (Fig. 9B). As expected, OA had a minimal toxic effect, compared with PA and LA.
Fig. 9. Cytotoxic effect of FFA in J774.2 macrophages. (A) J774.2 macrophages were exposed to BSA-complexed PA (150 µM) for 12 or 24h, then viability was measured as described in "Materials and methods". Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the previous time exposure, \( P<0.05 \). (B) J774.2 macrophages were exposed to different concentrations of LA and OA for 48h. Viability was measured as described in "Materials and methods". Each dot on the curve represents average ± SD (n=3). *, indicates significant statistical difference from the untreated control group.

Morphological changes used for apoptosis evaluation include decline in cell size and formation of apoptotic bodies. In contrast, necrosis is characterized by early rupture of cellular membranes and swelling of cells. As compared to untreated cells (Fig. 10A), in macrophages exposed for 48h to soybean TG (Fig. 9B) or olive oil-based TG (Fig. 10C), increased size and developed extensions and blebs were observed. Moreover, the treatment increased cellular roughness.

Exposure of the cells to PA (Fig. 10D), OA (Fig. 10E) or LA (Fig. 10F) decreased cell size and the formation of apoptotic bodies was observed. No extension development was observed in FA-induced changes of cellular shape.
Fig. 10. Changes in cellular morphology following exposure of J774.2 macrophages to lipid treatments. Light microscopy, x40. (A) Untreated cells; (B) Macrophages treated with soybean TG (1 mg/ml) for 48h; (C) Macrophages treated with olive TG (1 mg/ml) for 48h. Arrows appoint on enlarged, rough cells with extensions; (D) Macrophages treated with PA (150 μM) for 24h; (E) Macrophages treated with LA (600 μM) for 48h; (F) Macrophages treated with OA (600 μM) for 48h. Arrows appoint on cells with decreased size and apoptotic bodies.
3.1.6 Effect of PA on intracellular TG accumulation, ROS generation and cell cycle

Lipids located in the cytoplasm may be stored in several ways, including protein-bound or membrane-anchored forms. However, the most neutral way of lipid accumulation is formation of intracellular TGs. Some types of FA are reported to increase TG formation while others do not (Listenberger et al., 2003; Martins de Lima et al., 2006). In this study, PA did not lead to TG accumulation within the J774.2 macrophages (Fig. 11A).

Caspase-3 activity was measured to determine the type of cellular death following PA treatment. PA led to caspase-3 activation (Fig. 11B). Measurement of cell cycle following 12h exposure of the cells to PA demonstrated that in our model PA-induced lipotoxicity was accompanied with G₂ cell cycle arrest (Fig. 11C), and not G₁ arrest as the effect of TG was observed. Moreover, time-dependent DNA fragmentation (cells in sub-G₁ phase) was observed in this process (Fig. 11D), supporting our findings of apoptotic death pathway induced by PA, which is different from the TG lipotoxic effect.
Fig. 11. Features of J774.2 macrophage death pathway following exposure of J774.2 macrophages to PA. (A) Intracellular TG content was measured using Nile Red probe, as described in "Materials and Methods". *, indicates significant statistical difference from the untreated control group, † - significant statistical difference from the previous time exposure, \( P<0.05 \). (B) Caspase-3 activity was measured using a fluorogenic probe by microfluorimeter, as described in "Materials and methods". Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, \( P<0.05 \). (C) The cells were treated with BSA-complexed PA (150 µM) for 12h, and cell cycle was measured as described in "Materials and methods" in untreated control (left) or PA-treated cells (right). *, indicates significant statistical difference from the untreated control group, \( P<0.05 \). (D) Sub-G₁ phase (fragmented DNA) as a result of treatment with PA (150 µM). Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the previous time exposure, \( P<0.05 \).
3.2 Aim 2: To elucidate the mechanism by which TG regulate oxidative stress and ROS production and the role of antioxidants in this pathway.

3.2.1 TG-induced oxidative stress in J774.2 macrophages

To study the effect of TG on cellular redox status, the level of cellular ROS was measured with the fluorescent marker H$_2$DCF-DA (Fig. 12A). FACS analysis showed that a 12 and 24h exposure to 1 mg/ml TG enhances ROS production in the cell population. Nitro blue tetrazolium reduction assay, did not demonstrate increase in superoxide production after exposure of the cells to LE for 12, 24 and 48h (Fig. 12B). Moreover, 48h of exposure culminated with a significant decrease in superoxide levels.

![Fig.12. Effect of TG on cellular ROS generation.](image)

J774.2 macrophages were incubated with TG (1 mg/ml) for 12, 24 and 48h. Control represents untreated cells seeded and kept in culture for 48 h. (A) ROS levels as measured with H$_2$DCF probe by flow cytometry. (B) Superoxide levels as measured with nitro blue tetrazolium (NBT), as described in "Materials and Methods". Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, $P<0.05$. 
To eliminate the possibility that the source of ROS was in LE hydroperoxides, peroxide value (PV) of soybean LE was measured, and a PV of 12 µmol/kg of LE was found. Then the cells were treated with TBH at the equimolar concentration of 12 µM (Fig. 13). No loss in viability was observed after 48h of exposure to TBH (Fig. 13). In comparison, exposure to LE for 48h caused a significant decrease in cell viability. Therefore, the lipotoxic effect of LE is probably not due to oxidized PUFA.

Fig.13. Effect of tert-butyl hydroperoxide (TBH) treatment on J774.2 macrophages. After determining a peroxide value (PV) of 12 mmol/kg triacylglycerol (TG), (or 12 µmol/kg LE) the J774.2 macrophages were exposed to an equimolar concentration of TBH (12 µM) for 12, 24 and 48 h, and its effect on cell viability was compared to exposure to lipid emulsion (LE). Control represents untreated cells seeded and kept in culture for 48 h. Bars represent mean ± SD (n=3). *, indicates significant statistical difference from the untreated control group, \( P<0.05 \).

Changes in the cellular antioxidant status occurred, reflecting oxidative stress. Measurements of GSH showed reduction in the levels of this antioxidant in the presence of high concentrations of ROS (Fig. 14).
Fig. 14. Effect of TG on intracellular reduced glutathione (GSH) concentration. GSH concentrations were measured as described in methods. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, $P<0.05$.

3.2.2 Kinetic association between ROS and cell death in J774.2 macrophages

Flow cytometry analysis showed that a 12h exposure to 1 mg/ml TG enhances ROS production in the cell population (Fig.15, right). However, after 24h exposure to TG, two cell populations (low and high ROS) could be distinguished. Most of the cells exhibited enhanced ROS levels, but a secondary population of cells had lower ROS levels than in controls. Cells with low ROS levels became dominant at 48h. Comparative analysis of DNA status (Fig.15, left) indicated 50% DNA fragmentation 48h after TG treatment. Therefore, ROS function is an early signal, appearing prior to activation of the endonucleases that degrade cellular DNA. DNA degradation occurred only as a late event together with loss of membrane integrity.
Fig. 15. Analysis of reactive oxygen species (ROS) levels and DNA fragmentation in J774.2 cells following exposure to TG. J774.2 macrophages were incubated with TG (1 mg/ml) for 12, 24 and 48 h. Control represents untreated cells seeded and kept in culture for 48 h. DNA fragmentation analysis (left histogram column) and ROS levels (right histogram column) were measured by flow cytometer, as described in methods. Each histogram is representative of three experiments.
For further understanding the interaction between TG, ROS and features of cell death, the pro-apoptotic protein-synthesis inhibitor cycloheximide (CH) was used. CH is known to activate cellular signaling, resulting in caspase activation and apoptosis (Schmitz et al., 2004). CH treatment alone decreased ROS production in the cells, while combined treatment with TG and CH did not lead to increased ROS generation, unlike TG treatment (Fig. 16A). Combined CH and TG treatment resulted in cell death after 48 h, indicating that protein-synthesis inhibition does not prevent the cell-death effect of TG (Fig. 16B). Pretreatment for 24h with CH led to increased caspase-3 activity (Fig 16C); however, TG treatment following CH pretreatment partially suppressed the caspase-3 activation (Fig. 16C). These results probably reflect a decrease in ROS generation in the cells following CH treatment, which allows caspase-3 activity in the higher reducing environment. Therefore, a higher oxidation state in lipotoxicity suppresses caspase-3 activity and intrinsic apoptosis capacity and may lead to necrotic cell death.

### 3.2.3 Effects of antioxidants in TG-induced lipotoxicity in J774.2 macrophages

The capacity of antioxidants to prevent lipotoxicity was evaluated. A thiol compound, N-acetyl-cysteine (NAC), was used to prevent ROS production and cell death (Fig. 17). TG-induced ROS production was suppressed by addition of 0.5 mM NAC (Fig. 17A). NAC also protected the cells against TG-induced cell death (Fig. 17B).
Fig. 16. Effect of TG on cycloheximide (CH)-treated cells. Control represents untreated cells seeded and kept in culture for 24 h for A, 48 h for B and C. (A) ROS measured following TG (1 mg/ml), CH (0.1 μM), and combined treatment. *, indicates significant statistical difference from the untreated control group, \( P<0.05 \). (B) Cell viability measured after TG, CH and combined treatment. *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the CH alone treatment, \( P<0.05 \). (C) Cells were pretreated with CH for 24 h and then exposed to LE for the next 24 and 48 h. Caspase-3 activity was measured as described in methods. Bars represent averages ± SD of three experiments. *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the CH alone treatment, \( P<0.05 \).
Fig. 17. **Antioxidant and protective effect of N-acetyl cysteine (NAC) in TG-induced lipotoxicity.**

Cells were treated with TG (1 mg/ml) in the presence or absence of NAC (0.5 mM) for 12 or 24 h. Control represents untreated cells seeded and kept in culture for 24 h for A, 48 h for B. (A) Measurements of ROS. *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the TG alone treatment, \( P<0.05 \). (B) Cell viability. Bars represent averages ± SD of three experiments (\( n = 3 \)), *, indicates significant statistical reduction from the untreated control group; § - significant statistical increase from the untreated control group; † - significant statistical difference from the TG alone treatment, \( P<0.05 \).

A series of additional antioxidants were screened for their ability to prevent lipotoxicity (Fig. 18A). Ascorbic acid (0.5 mM) and resveratrol (0.2 mM) significantly decreased the level of cell death. Other water- and lipid-soluble antioxidants, such as quercetin, lycopene, beta-carotene, alpha-tocopherol, selenium and racemic lipoic acid, did not have any protective effect or even increased cell death levels. When the protective antioxidants NAC, ascorbic acid and resveratrol were examined together at one-tenth their concentrations used in the previous experiment
(more closely approximating bioavailable levels), a synergistic protective effect was observed (Fig. 18B). These combined compounds afforded full protection from TG-induced lipotoxicity.

Fig. 18. **Antioxidant protection from TG-induced lipotoxicity.** Control represents untreated cells seeded and kept in culture for 48 h. (A) Cells were treated with TG (1 mg/ml) in the presence or absence of the following antioxidant compounds: ascorbic acid (AA, 0.5 mM), resveratrol (resv, 0.2 mM), quercetin (querc, 0.2 mM), lycopene (lyc, 10 µM), beta-carotene (carot, 10 µM), alpha-tocopherol (toc, 0.2 mM), lipoic acid (LA, 0.25 mM) and selenium (Se, 1 µM). *, indicates significant statistical reduction from the untreated control group; † - significant statistical reduction from the TG alone-treated cells, §-significant protective effect of the antioxidant, P<0.05. (B) Cells were treated with TG (1 mg/ml) in the presence or absence of: ascorbic acid (50 µM), NAC (50 µM) or resveratrol (20 µM), or their combination. n =3, *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from exposure to another antioxidant, P<0.05.
3.2.4 Effect of FA on intracellular ROS generation and antioxidant protection

To compare the influence of TG on ROS generation and the protective role of antioxidants with this FA-induced effect, ROS generation following exposure to PA, LA and OA was evaluated. No increase in ROS production was observed after these treatments (Fig. 19 A,B). Comparison of the effect of an antioxidant NAC in TGs and FA-induced lipotoxicity demonstrated their differential effects on the cell death process. NAC protected from TG-induced lipotoxicity, but did not protect from the FA-triggered pathway of PA, OA and LA (Fig. 19C).
Fig. 19. Effect of FA on intracellular ROS generation in J774.2 macrophages. (A) ROS generation in J774.2 macrophages after their treatment with PA (150 µM). (B) ROS generation in J774.2 macrophages after their treatment with TG (1 mg/ml), OA (600 µM) or LA (600 µM) for 24h. ROS were measured as described in "Materials and methods". Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group. (C) J774.2 macrophages were treated either with TG (1 mg/ml) for 48h, or with OA or LA (600 µM) for 48h, or with PA (150 µM) for 24h with or without NAC (0.5 mM) which was added simultaneously with lipid treatments. Bars represent averages ± SD (n=3). Cellular viability was measured as described in "Materials and methods". Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the same lipid exposure without NAC, P<0.05.
3.3 **Aim 3:** To investigate the effect of TG on genes involved in mitochondrial 
biogenesis and mitochondrial function.

3.3.1 **Identification of the source for generation of ROS induced by TGs in J774.2 
macrophages**

In order to identify the intracellular source of the TG-induced ROS and to interrupt 
the steady-state production of ROS, the cells were treated with rotenone, a 
mitochondrial complex I inhibitor. Rotenone was added to the LE-treated cells 5 min 
before their 30-min incubation with the H$_2$DCF-DA probe. Inhibition of complex I 
significantly decreased ROS production in TG-treated cells (Fig. 20). These data 
indicate that ROS were generated by an endogenous cellular source and are not the 
result of oxidized lipids delivered from the cell-culture medium or intracellular lipid 
oxidation. Moreover, these data show that the ROS in TG-treated cells are mostly 
generated in mitochondria.
Fig.20. **Effect of mitochondrial complex I inhibitor rotenone on ROS generation in TG-treated J774.2 macrophages.** The cells were incubated with soybean TG (1mg/ml) for 24 h. Rotenone (40 µM) was added to the control and LE-treated cells 5 min prior to adding H₂DCF-DA and 30 min incubation. ROS levels were measured by flow cytometry. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from the treatment without rotenone, P<0.05.

### 3.3.2 ATP as an indicator of mitochondrial function in TG-treated J774.2 macrophages

To further investigate the effect of TGs on mitochondrial function, measurement of intracellular ATP content was undertaken, demonstrating that at 24h treatment with TG, cellular synthesis of ATP drops to a significantly low level (Fig. 21), indicating reduced activity of mitochondrial electron transfer chain (ETC).
Fig. 21. **Effect of TG on mitochondrial function.** The cells were incubated with soybean TG (1mg/ml) for 24 h. ATP was measured by bioluminescence method. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, $P<0.05$.

### 3.3.3 Effect of TGs on mitochondrial complexes in J774.2 macrophages

As shown in Fig. 20, mitochondrial complex I inhibitor rotenone prevents elevation in intracellular ROS levels, indicating an impairment in mitochondrial structure. To test this hypothesis, the content of mitochondrial complexes in the cells was evaluated by SDS-PAGE. Three subunits of complex I were evaluated, NDUFA9, NDUFA10 and NDUFS3. All of these subunits are nuclear-encoded, and for all of them the reduction in expression was observed (Fig.22). NAC prevented these TG-induced changes in complex I subunits.
Fig.22. Effect of TGs and NAC on expression of mitochondrial complex I subunits in J774.2 macrophages. The cells were incubated with soybean TG (1mg/ml) for 24 h in presence or absence of NAC (0.5 mM). The protein content was evaluated by SDS-PAGE and Western blot, as described in "Materials and Methods". Quantitative evaluation was presented using "Gelpro" densitometry program. (A) Content of NDUFA9 subunit; (B) Content of NDUFA10 subunit; (C) Content of NDUFS3 subunit. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, P<0.05.
For a representative evaluation of mitochondrial complex III, a subunit UQCRC2 was chosen. This subunit is also a nucleus-encoded one, and, similar to complex I subunits, its expression in TG-treated cells was reduced. NAC also affected the UQCRC2 expression at the same way as complex I subunits, preventing the down regulation of its expression (Fig.23).

![UQCRC2 and beta-actin Western blot](image)

**Fig.23. Effect of TGs and NAC on expression of mitochondrial complex III subunit UQCRC2 in J774.2 macrophages.** The cells were incubated with soybean TG (1mg/ml) for 24 h in presence or absence of NAC (0.5 mM). The protein content was evaluated by SDS-PAGE and Western blot, as described in "Materials and Methods". Quantitative evaluation was presented using "Gelpro" densitometry program. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, \( P<0.05 \).

Complex IV was evaluated using a subunit Cox 4, which is also nucleus-encoded. The subunits of cytochrome oxidase have a complex mechanism of incorporation into the inner mitochondrial membrane (Herrmann and Funes, 2005). No changes in the content of 17 kDa protein CoxIV was observed following TG treatment, however, the
exposure to TGs for 24h led to a consistent appearance of a 20-25 kDa protein, which could be a Cox 4 precursor (Fig.24A) (Cannino et al., 2004). This band was analyzed separately, including combined treatment with TGs and NAC, thus preventing the increase in this precursor accumulation (Fig.24B).

![Fig.24. Effect of TGs and NAC on expression of mitochondrial complex IV subunit Cox 4 in J774.2 macrophages.](image)

The cells were incubated with soybean TG (1mg/ml) for 12 and 24 h in presence or absence of NAC (0.5 mM). The protein content was evaluated by SDS-PAGE and Western blot, as described in "Materials and Methods". Quantitative evaluation was presented using "Gelpro" densitometry program. (A) Cox 4 subunit and its precursor. (B) Analysis of accumulation of Cox 4 precursor in presence or absence of NAC. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, $P<0.05$. 

![Graph showing densitometry values for Cox 4 precursor and beta-actin with statistical significance indicated.](image)
Changes in content of mitochondrial complexes were evaluated also in MPM cells. Reduction in complex I subunits (Fig. 25A,B) and appearance of a Cox 4 precursor band were observed in MPM (Fig. 25C) as well as in the J774.2 macrophages.

Fig. 25. Effect of TGs on expression of mitochondrial complexes in MPM. The cells were incubated with soybean TG (1mg/ml) 24 h. The protein content was evaluated by SDS-PAGE and Western blot, as described in "Materials and Methods". Quantitative evaluation was presented using "Gelpro" densitometry program. (A) NDUFS3 subunit of complex I (B) NDUFA9 subunit of complex I. (C) Cox 4 subunit and its precursor of complex IV. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group, $P<0.05$. 

3.3.4 Effect of mitochondrial complex inhibitors on TG-induced lipotoxicity in J774.2 macrophages

To evaluate the role of mitochondrial impairment in TG-induced impaired cell viability, two inhibitors of mitochondrial complexes were used: rotenone for complex I and myxothiazol for complex III. Concentrations of the inhibitors were calibrated to non-toxic doses. J774.2 macrophages were treated with TGs in presence or absence of the complex inhibitors. Each inhibitor demonstrated significant protection from reduction in viability in soybean TG-treated cells (Fig.26), thus corroborating the hypothesis that mitochondrial-generated ROS are a key point in the mechanism of TG-induced lipotoxicity.

![Graph showing the effect of inhibitors on TG-induced lipotoxicity.](image)

**Fig.26. Effect of inhibitors of mitochondrial complexes on TG-induced lipotoxicity.** The cells were incubated with soybean TG (1mg/ml) for 24 h in presence or absence of mitochondrial complex I inhibitor rotenone (0.1 μM) and complex III inhibitor myxothiazol (25 nM). Bars represent averages ± SD (n=3). *, indicates significant statistical reduction from the untreated control group; † - significant statistical reduction from non-TG treatment; § - significant protection from TG-induced cell death, $P<0.05$. 
3.3.5 Effect of TGs on the nuclear factors associated with the mitochondrial biogenesis

PGC-1 family proteins are known as responsible for mitochondrial biogenesis. To study the nuclear factors involved in the TG-induced impairment in mitochondrial biogenesis, the expression of PGC-1 family proteins was evaluated at the level of mRNA, using a real-time PCR technique. Treatment with soybean TG for 12h initiated reduction in PGC1-alpha expression (Fig. 27A), while the expression of PGC1-beta remained unchanged (Fig. 27B), indicating that TGs affect the mitochondrial biogenesis through PGC1-beta independent pathway. The RNA expression of PGC1-alpha downstream protein, NRF-1, was also evaluated, and showed reduction in 24h (Fig. 27C). NAC demonstrated a complete protection from TG-induced reduction at 12h time point (Fig. 27D).
Fig. 27. Effect of TG on mRNA expression of nuclear factors responsible for mitochondrial biogenesis. J774.2 macrophages were treated with soybean TG (1mg/ml) for 12 and 24h. mRNA was evaluated by real-time PCR assay, as described in "Materials and Methods". (A) PGC-1 alpha mRNA expression; (B) PGC-1 beta mRNA expression; (C) NRF-1 mRNA expression. (D) Cells were treated with soybean TG (1mg/ml) for 12h in presence or absence of NAC (0.5 mM) and mRNA expression was measured. Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from previous time exposure, P<0.05.
To investigate the mechanism by which NAC protects at an early time point, even before ROS are elevated, evaluation of lipid content by Nile Red was performed in cells treated with TGs in presence or absence of NAC. Prevention of intracellular TG accumulation was observed in combined treatment with TG and NAC (Fig.28), suggesting a critical role of oxidative stress in uptake of TGs either due to total intracellular stress, or redox state of receptor responsible for the TG uptake by the cells.

Fig.28. Preventive effect of NAC on LE-induced intracellular TG accumulation. J774.2 macrophages were treated with soybean TG (1mg/ml) for 24 and 48h in presence or absence of NAC. Nile Red assay was performed as described in "Materials and Methods". Bars represent averages ± SD (n=3). *, indicates significant statistical difference from the untreated control group; † - significant statistical difference from previous time exposure, § - significant statistical difference from TG alone treated cells at the same time exposure, P<0.05.
4. Discussion

4.1 TG-induced impairment in cell viability in macrophages

Involvement of impaired lipid metabolism in such abnormalities as metabolic syndrome, diabetes, fatty liver and atherosclerosis emphasizes the importance of lipotoxic effects of different lipid molecules in cells, tissues and organisms. The association between disturbances in lipid metabolism and atherosclerosis is well described for cholesterol, fatty acids and several lipoprotein particles. Atherosclerotic plaques contain inflammatory and immune cells (mainly macrophages and T cells), as well as vascular endothelial cells, smooth muscle cells, extracellular matrix, lipids and acellular lipid-rich deposits (Hansson and Libby, 2006). Following massive lipid uptake, macrophages turn into foam cells, which are the most common cells in early atherosclerotic plaques. In mature plaques, foam cells and extracellular lipid droplets form a core region that is surrounded by a cap of smooth muscle cells and a collagen-rich matrix. Several phenotypes of macrophages are found in plaques, including inflammatory macrophages and also foam cells. Development of foam cells is characterized by an accumulation of cholesteryl esters in macrophages (Jonasson et al., 1986). In our experiments it was shown that uptake of TG particles by macrophages potentiates foam cell formation and death.

In our study, both MPM and J774.2 macrophages demonstrated the ability to uptake TGs from LE (Fig. 1, 2). The uptake of TGs by macrophages has been demonstrated through a coated pit-dependent mechanism (Carvalho et al., 2002). Experiments with a broad spectrum lipase inhibitor PO showed that the uptake and the accumulation of intracellular macrophage TGs was largely independent of the activity of lipases (Fig.3B).
The results of our study indicate that TGs induce a unique pathway of lipotoxicity in macrophages, which can be initiated independently of the cellular degradation of TGs to FFA, and involves oxidative stress and mitochondrial dysfunction. The toxicity of increased concentrations of FFA in cells is well documented and can be attenuated to some extent by incorporation of the FFA into intracellular TGs (Martins de Lima et al., 2006; Martins de Lima et al., 2007). However, in the current study, toxic effects of TG uptake were characterized.

The characterization of TG-induced macrophage cell death demonstrated suppression of caspase-3 activity, lack of flip-flop of phosphatidyl serine molecule (Fig. 5), and DNA fragmentation as a late event of the death pathway (Fig. 15). Morphology of dead TG-treated macrophages was like those of necrotic death (Fig. 10B). These findings led us to the conclusion that the TG-induced cell death was necrotic, or oncotic.

Cellular death was not prevented by PO (Fig. 4C) proving that TGs did not have to be degraded to FA and glycerol to induce cell death. Thus, the TG molecule played a direct role in TG-induced lipotoxicity. Although PO may not prevent the release of small amounts of FFA from TGs, the toxic effect due to FA release would have resulted in different morphological features, similar to those observed for purified FA.

The study of cytotoxicity of FFA is controversial. Apoptotic death has been previously linked to PA exposure (Cacicedo et al., 2005; Landau et al., 2006; Wei et al., 2006). Ulloth et al. showed that neuronal cell death with apoptotic features followed cellular exposure to palmitic and stearic acids (Ulloth et al., 2003). Moreover, there is evidence that both oleic and linoleic BSA-complexed acids protect from PA-induced lipotoxicity in equimolar concentrations, completely preventing death in several cell types (Beeharry et al., 2004; Listenberger et al., 2003). In our
study, death induced by PA in macrophages was accompanied by apoptotic features including caspase-3 activation, DNA fragmentation and morphological changes, such as decreased cell size and condensation of DNA (Fig. 9A, 10D, 11). Moreover, this work supports a series of studies which observed low levels of toxicity in cells following exposure to oleic acid (Moffitt et al., 2005; Ulloth et al., 2003). In contrast to the low toxic effect of oleic acid (Fig. 9B), olive oil TG, which is comprised of 60% oleic acid, was found to be toxic in macrophages, leading to cell cycle and morphological changes in macrophages similar to those of soybean oil TG (Fig. 6, 7, 10C), but distinct from OA (Fig. 10F).

Although changes in cell cycle may be of less physiological relevance for plaque infiltrating macrophages, they could demonstrate common and distinct points in lipotoxic pathways of different lipid molecules. Thus, TG-induced pathway involved G₁ arrest in both soybean and olive oil-base TG treatments (Fig. 7), while PA-treated cells demonstrated G₂ arrest (Fig. 11C).

### 4.2 Role of ROS in TG-induced lipotoxic pathway in macrophages

In our model, the lipotoxic pathway initiated by TGs was characterized by elevated H₂DCF-DA positive ROS generation following 12 and 24h of exposure (Fig.12A), while treatment with FA did not lead to increase in ROS (Fig.19A,B). ROS from mitochondria and other cellular sources have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA (Freeman and Crapo, 1982; Thannickal and Fanburg, 2000). Accumulating evidence suggests that ROS are not only injurious by-products of cellular metabolism but also essential participants in cell signaling and regulation (Rhee, 1999; Thannickal and Fanburg, 2000).
TG-induced oxidative stress and increased steady-state ROS production at 12 and 24h correlated with caspase-3 inhibition. Increased oxidative tension in cells can lead to caspase-3 activation and apoptosis. However, further increases in ROS levels by exogenously added hydrogen peroxide block the caspase system, which needs a reducing environment to function (Chandra et al., 2000). An additional experiment was undertaken to show how TGs affect apoptotic cells (Fig. 16). For this purpose, J774.2 macrophages were exposed to a proapoptotic agent cycloheximide (CH). Prolonging ROS production attenuated caspase activity and shifted apoptosis to necrosis. While CH induced apoptosis accompanied by caspase-3 activation in the macrophages, TGs attenuated this caspase-3 activity in CH-treated cells, thus indicating that TGs have the potential to change death pathways from apoptosis to necrosis.

Additional evidence of the role of ROS as signaling molecules was observed in the kinetic changes in ROS generation as compared to kinetics of DNA fragmentation. The damage to DNA was observed only after reduction in ROS production, which implies that a signaling pathway was initiated by ROS (Fig. 15).

In comparison with the extracellular environment, the cytosol is normally maintained under reducing conditions. This is accomplished by the "redox-buffering" capacity of intracellular thiols, primarily GSH. Accumulating evidence suggests that, in addition to its "antioxidant" functions, GSH participates in cell signaling processes. Cellular GSH depletion has been found to be associated with decreased cell proliferation in vascular endothelial cells (Das et al., 1992; White et al., 1992), and increased proliferation of fibroblasts (Thannickal and Fanburg, 2000).

The protective influence of NAC found in our work (Fig. 17), suggests the critical role of GSH in TG-induced lipotoxic pathway. This effect of NAC, which is a
precursor of glutathione, indicates an interaction at the level of water-soluble antioxidants. The specificity of NAC protection from death of macrophages caused by TGs and lack of such an effect in FA-initiated pathway (Fig. 19D) shows that different lipid molecules affect macrophages in different ways. In the TG-induced pathway, NAC provided protection from ROS elevation and cell death, as well as from mitochondrial damage and change in the status of factors responsible for mitochondrial biogenesis.

In addition to NAC, the antioxidants ascorbic acid, and resveratrol played a protective role in TG-induced lipotoxicity (Fig. 18). Experimental evidence shows that GSH can spare ascorbate and, conversely, ascorbate can spare GSH (Jain et al., 1992; Martensson et al., 1991). Ascorbic acid has the potential to protect macrophages from apoptosis (Asmis and Begley, 2003) and to recycle phenolic antioxidants. Resveratrol, a phenolic compound, is relatively water soluble and is completely dissolved in aqueous media at high micromolar concentrations. This suggests that other water-soluble phenolic antioxidants may be effective as well. A combination of these compounds at lower concentrations which are similar to physiological levels, exhibited a synergistic effect in protecting from necrotic death induced by TG, implying that there are probably distinct points of protection in this death pathway. Interesting, we could not identify any lipid-soluble antioxidant with the potential to attenuate the rate of TG-induced cell death.

4.3 Mitochondrial impairment in TG-induced lipotoxic pathway in macrophages

We show that TGs impair mitochondrial functions in macrophages. Because the mitochondrial complex I inhibitor rotenone was able to prevent the majority of ROS production (Fig.20), and because rotenone and myxothiazol partly protected from TG-
induced lipotoxicity in this study (Fig. 26), it strongly suggests that a mitochondria are responsible for elevated ROS levels. Several sources of ROS production occur in phagocytic cells during respiratory burst, and the specific source of ROS generation in the pathway of TG-induced lipotoxicity was of high pertinence to our study. It is well known that, in addition to mitochondrial complexes, NADPH oxidase also produces ROS which contribute to changes in cellular redox state (Bokoch and Zhao, 2006).

Analysis of mitochondrial functions demonstrated ATP depletion as a result of TG treatment (Fig. 21), which could be explained by depletion of complex I subunits, NDUFA9, NDUFA10 and NDUF3. The reduction in complex I subunits was common in J774.2 and MPM cells (Fig. 22, 25A,B). Complex I (NADH–ubiquinone oxidoreductase) is the most frequently affected complex of the oxidative phosphorylation (OXPHOS) system (Smeitink et al., 2001). The enzyme couples the transfer of two electrons from NADH to ubiquinone for the translocation of four protons across the mitochondrial inner membrane. The generated proton gradient is used by complex V to produce ATP. Deficiency in several complex I subunits has a potential to lead to disturbances in enzyme assembly (Ugalde et al., 2004b).

We observed a reduction in the complex 3 subunit analyzed in our study (Fig. 23), which could be explained by the TG-induced deficiency of complex I. One study in human skin fibroblasts taken from complex I-deficient patients found that mutations of NDUFA9 and NDUF3, among other subunits, disable general assembly and stability of complex I (Ugalde et al., 2004a). The same study showed that different mutations in complex I genes affect the stability of other OXPHOS complexes, and lead to a specific decrease of intact mitochondrial complex 3 in patients with mitochondrial disorders.
The expression of analyzed subunit Cox 4 did not change, however, a protein which could be referred to as a Cox 4 precursor (Cannino et al., 2004) accumulated both in J774.2 and in MPM cells (Fig. 24, 25C). This accumulation could be explained by disturbances in complex IV incorporation into the inner mitochondrial membrane. The cytochrome c oxidase is the terminal enzymatic complex of the respiratory chain in eukaryotes. It couples the transfer of electrons between cytochrome c and molecular oxygen to translocate protons across the inner membrane of the mitochondria. The assembly line for Cox subunits has a complex mechanism which requires translocation of nucleus-encoded subunits through the inner membrane of the mitochondria and their consequent insertion into the complex. This process demands several additional factors such as copper and protein complexes (Herrmann and Funes, 2005).

The changes in expression and incorporation mechanisms of the mitochondrial complexes suggest involvement of nuclear factors responsible for mitochondrial biogenesis in this lipotoxic pathway. PGC-1 family proteins are now being widely studied in association with metabolic tissue, such as adipose tissue, liver, heart and muscle (Feige and Auwerx, 2007) because of their connection to regulation of energy metabolism and mitochondrial biogenesis. To date, less PGC-1 proteins expression has been observed in cases of obesity and diabetes. Because expression of both PGC-1 alpha and -beta is reduced in skeletal muscle from prediabetic and diabetic humans (Richardson et al., 2005), PGC-1 has emerged as a potential candidate gene mediating diabetes-related metabolic phenotypes. Infusion of lipids decreases expression of PGC-1 alpha and nuclear-encoded mitochondrial genes in muscle (Mootha et al., 2003). Conversely, severe caloric restriction and exercise training, which improves
insulin sensitivity, increases PGC-1 alpha expression in skeletal muscle of obese subjects (Richardson et al., 2005).

The role of PGC-1 in macrophages has not been yet investigated. In our study, we observed lipotoxic effects of TGs, mediated by mitochondrial dysfunction in macrophages. Thus, PGC-1 alpha and beta were investigated (Fig. 27 A,B). The results demonstrated reduction of mRNA of PGC-1 alpha levels, while no change in PGC-1 beta was observed. PGC-1 alpha downstream transcription factors are responsible for mitochondrial biogenesis. NRF-1 regulates factors involved in mitochondrial DNA synthesis and also factors required for production of nuclear-encoded subunits of mitochondrial complexes (Scarpulla, 2006). Our results showed that the levels of mRNA of NRF-1 were reduced (Fig. 27C) as a response to a decrease in PGC-1 alpha, explaining the effect on complex I and III synthesis.

The ability of NAC to protect from the changes observed in mitochondrial complexes and nuclear factors responsible for mitochondrial biogenesis could be explained by the fact that in mammalian cells GSH is distributed in the cytoplasm, the mitochondria and also in the nucleus. Due to the lack of de novo GSH synthesis in mitochondria, it has been suggested that GSH is transported from the cytoplasm to the mitochondrial matrix via an ATP-dependent transporter located on the mitochondrial membrane (Zhu et al., 2007). Thiol groups are involved in OXPHOS machinery, particularly in complex I and IV activities, and also in the maintenance of mitochondrial membrane integrity (Nicoletti et al., 2005; Yagi and Hatefi, 1984). NAC has been reported as an agent able to enhance complex I activity (Martinez Banaclocha, 2000).

The prevention of intracellular TG accumulation following exposure of cells to combined treatment with TGs and NAC (Fig. 28) suggests that this prevention was
caused by NAC protection from TG-induced lipotoxicity. However, the protective
effect of NAC in a synergistic manner with ascorbic acid and resveratrol at
concentrations which each individual compound does not affect the lipotoxic process,
points to the involvement of NAC in an antioxidative protective pathway.

4.4 Summary and conclusions

1) Extracellular TGs from LE accumulate in J774.2 and in MPM cells without
undergoing hydrolysis to FFA and induce lipotoxic pathway which culminates
in necrotic cell death of macrophages.

2) ROS play a pivotal role in TG-induced lipotoxic pathway. Water-soluble
antioxidants have the potential to protect macrophages from TG-induced cell
death. The GSH system may be a key point in prevention of TG-induced
lipotoxicity.

3) The main source of ROS in this pathway is mitochondria, and impairment in
biosynthesis and assembly of mitochondrial complexes play a key role in the
lipotoxic effect of TGs.

Taken together, the results lead us to suggest a pathway which is induced by TGs
in macrophages. This pathway is demonstrated in Scheme 1.
Scheme 1. **The pathway of TG-induced lipotoxicity in macrophages.** TGs are taken up by macrophages and cause increased ROS generation, which decreases the levels of intracellular GSH, and, probably impairs cellular thiol tone. Another effect of ROS is a direct damaging of mitochondrial ETC proteins. High ROS generation induces nuclear response of PGC-1 alpha and NRF-1, followed by impaired synthesis and assembly of mitochondrial proteins and by additional mitochondrial damage. The signal transduction results in supression of caspase-3 activity, formation of foam cells and necrotic cell death. Antioxidative capacity of NAC, ascorbic acid and resveratrol is protective from ROS damage. Another way of action of NAC is preventing of intracellular TG accumulation.
5. Bibliography


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