

# **Is nitric oxide a mediator in fatty acid-induced $\beta$ -cell apoptosis?**

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**Ermin Basic**  
**Class H-02**

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## **Abstract**

This paper reviews 15 articles about nitric oxide's role in FFA-induced apoptosis/cell death of  $\beta$ -cells in relation to DM2. These are mostly in vitro studies, but also included are 3 in vivo experiments. The main objects are to see if nitric oxide is a mediator in FFA-induced apoptosis/cell death, and if mechanisms are the same as in cytokine-induced apoptosis, like in Diabetes type 1.

Results: In all the reviewed studies it is demonstrated that FFAs induce apoptosis, in vitro by exposing cells and islets to FFAs, as well as in vivo by measuring apoptosis in islets of obese rats and islets of wild-type rats. It is also shown in vitro that unsaturated fatty acids are less cytotoxic to  $\beta$ -cells than saturated fatty acids, by inducing little or no cell death. After exposure to FFAs, cells and islets accumulated triglycerides. In vivo studies showed that administration of iNOS inhibitors prevent DM2 and  $\beta$ -cell loss in ZDF rats. Several in vitro studies showed that FFAs induce NO production via iNOS and that iNOS inhibitors attenuate cytotoxic effects of FFAs, whereas other studies showed no such effects.

Conclusion: FFAs induce apoptosis in  $\beta$ -cells and pancreatic islets. Saturated FFAs are more cytotoxic to  $\beta$ -cells and islets than unsaturated FFAs. FFAs induce accumulation of triglycerides in  $\beta$ -cells and islets. NO may be a mediator of FFA-induced apoptosis in  $\beta$ -cells, but does not seem to have the exact same induction pathways and damage methods as in cytokine-induced apoptosis.

## **Introduction**

Obesity is associated with several conditions, the most devastating of which may be type 2 diabetes. At the end of 2000 171 million humans were estimated to have diabetes, and this is expected to increase to 366 million by year 2030 (1). The mechanisms by which obesity leads to the development of this disease have been investigated intensively the last

decade. There are two aspects of the pathogenesis; insulin-resistance on one hand and  $\beta$ -cell loss and dysfunction on the other (2). The number of  $\beta$ -cells is reduced by 50% when diabetes is discovered (3). Most investigators have employed palmitate and/or oleate as the fatty acids of choice. This is because these two fatty acids represent the major species present in human serum; thus they are the principal molecules to which  $\beta$ -cells might be exposed in vivo (4). One of the major theories that have been investigated is the NO-mediated apoptosis. This is because NO is widely accepted to play a role in the death of  $\beta$ -cells exposed to pro-inflammatory cytokines involved in the development of type 1 diabetes (5). Here I am reviewing 15 articles that deal with NO and FFA-induced apoptosis/cell death. The aim is to find out if NO is a mediator in FFA-induced apoptosis in the same way as in cytokine-induced apoptosis.

## Methods

This is a review paper, which is based on many original articles. The main method to find articles was through the use of the database Pubmed. The search words I used were: (NEFA OR FFA OR free fatty acids OR fatty acids) AND (nitric oxide) AND (beta-cell OR beta-cells), which was interpreted by PUBMED like this: (("nonesterified fatty acids"[Text Word] OR "fatty acids, nonesterified"[MeSH Terms] OR NEFA[Text Word]) OR FFA[All Fields] OR ("nonesterified fatty acids"[Text Word] OR "fatty acids, nonesterified"[MeSH Terms] OR free fatty acids[Text Word]) OR ("fatty acids"[MeSH Terms] OR fatty acids[Text Word])) AND ("nitric oxide"[MeSH Terms] OR nitric oxide[Text Word]) AND (((("insulin-secreting cells"[TIAB] NOT Medline[SB]) OR "insulin-secreting cells"[MeSH Terms] OR beta-cell[Text Word]) OR beta-cells[All Fields])). 53 articles came up, and by going through them I picked out those relevant for the paper.

In addition to Pubmed I used other review articles (2, 6, 7) to get to their references, so that I could pick up relevant articles that I hadn't found in Pubmed with my search.

## Results

Here I will present the results from the 15 articles chosen. Only results relevant to the topic of the paper will be presented. **Results are presented in larger italics writing and methods in smaller normal writing. The smaller does not need to be read in order to understand this paper, and it can be skipped while reading.** It describes in more detail methods and conditions used for each result obtained.

Article 1. Rachek L.I., Thornley N. P., Grishko V. I., Ledoux S. P., Wilson G. L. (2006) Protection of INS-1 cells from free fatty acid-induced apoptosis by targeting hOGG1 to mitochondria. Diabetes 55, 1022-1028.

Aim: To examine whether targeting the DNA repair enzyme, human 8-oxoguanine DNA glycosylase/apurinic lyase (hOGG1) to mitochondria of INS-1 cells can prevent FFA-induced mtDNA damage and thus protect against FFA-induced apoptosis.

In this study INS-1 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and other standard additions. Cells were transfected with either pcDNA3.neo/mitochondrial targeting sequence (MTS)-OGG1 or empty vector (pcDNA3.neo) DNA. hOGG1 is a DNA repair enzyme that has glycosylase activity for the mutagenic 8-oxoguanine and lyase activity for abasic sites and 5'-deoxyribose phosphate. For experiments, cells were seeded into appropriate culture dishes 2-3 days before experiments. Long-chain FFAs (2:1, oleate:palmitate) were dissolved in 50% ethanol and exposed to INS-1 cells. BSA

(Bovine serum albumin) at a final concentration of 2% was added to the culture medium during FFA treatment.

## Results:

*Nitrite production by Wild-type, vector and MTS-OGG1 INS-1 cells and by the cell-free medium exposed to FFAs was increased compared to their respective control experiments without FFAs. But production of nitrite was significantly greater in all 3 cell types compared to the cell-free medium exposed to FFAs. Mean of 3 independent experiments.*

Cells and cell-free medium were exposed to 2 mmol/l FFAs for 6 h. Control cells received drug diluent only. Nitrite production was evaluated using Griess reaction.

*iNOS expression in Wild-type, MTS-OGG1- and vector-only-transfected INS-1 cells was induced after 6 h of exposure to 2 mmol/l FFAs. Mean of 3 independent experiments.*

All 3 cell types were exposed to 2.0 mmol/l FFAs for 6 h. Control cells received drug diluent only. Cells were then lysed and Western Blot was performed on the cytosolic protein fractions.

*mtDNA damage was increased after exposure of 2 mmol/l for 6 h in both cells, but the increase was significantly lower in MTS-OGG1-transfected cells.*

Cells were lysed, total DNA isolated, and quantitative Southern hybridizations were performed using a mtDNA-specific probe.

*Cell viability in vector-only-transfected and in MTS-OGG1 INS-1 cells was reduced after exposure to 2 mmol/l FFAs for 24 and 48 h. Cell viability was significantly higher in MTS-OGG1 than in vector-only-transfected cells. Mean of 3 experiments.*

Cells were treated with 2 mmol/l FFAs for 24 and 48 h. Control cultures were exposed to drug diluent only. 24 and 48 hours later, trypan blue was added and viable cells were counted using light microscopy.

*DNA fragmentation, a marker of apoptosis, in vector-only-transfected cells and MTS-OGG1 cells after treatment with FFAs for 9 h was increased compared to untreated controls. The fragmentation was lower in MTS-OGG1 cells. Mean of 3 independent experiments.*

Cells were treated with 2 mmol/l FFAs for 9 h. The presence of fragmented nuclear DNA in the cytoplasmic fraction of cell lysates was assessed by measuring DNA associated with nucleosomal histones using a specific two-site ELISA with an anti-histone primary antibody and a secondary anti-DNA antibody.

Conclusion: The results indicate that mtDNA damage plays an important role in FFA-induced apoptosis and that this might be mediated through increased amount of NO. Targeting DNA repair enzymes to mitochondria could be a potential therapeutic strategy for preventing or delaying the onset of DM type 2.

Article 2. Grishko V., Rachek L., Musiyenko S., LeDoux S.P., Wilson G. L.

(2005) Involvement of mtDNA damage in free fatty acid-induced apoptosis. Free Radical Biology & Medicine 38, 755-762

Aim: To investigate FFAs capacity to damage mtDNA in INS-1 cells and elucidate the mechanisms through which this damage occurred.

In this study INS-1 cells were used. The cells were grown at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum and other standard additions. Cells were seeded in dishes 2-3 days prior to experiments. BSA at a final concentration of 2% was added to the culture media during FFA treatment. Long-chain free fatty acids (2/1 oleate/palmitate) were dissolved in 50% ethanol and used for treatment of INS-1 cells.

Results:

*In cultures treated with 2mM FFA,  $34 \pm 3\%$  of the cells were apoptotic. When 0.5 aminoguanidine was added to the cells together with the FFA, the percentage of apoptotic*

*cells was reduced to  $5 \pm 0.08\%$ . Control cultures, as well as cultures treated with aminoguanidine only, did not have any apoptotic cells. Mean of 5 experiments.*

Cells were treated with 2 mM FFA for up to 24 h. At the selected times cells were fixed and stained with 4,6-diamidino-2-phenylindole(DAPI, 1 $\mu$ g/ml) for 30 min. Stained cells were examined by fluorescent microscopy to identify apoptotic cells, that were morphologically defined by cytoplasmic and nuclear shrinkage, and by chromatin condensation or fragmentation. 5 fields containing 40-50 cells each were used to calculate the percentage.

*Nitrite concentrations were elevated following FFA treatment, reaching a maximum value after 6 h. 0.5mM of aminoguanidine blocked the formation of nitrite.*

The Griess reagent was added to 100 $\mu$ l aliquots of medium, and the optical densities were measured at 555nm in a microplate reader after 10 min incubation at room temperature. Background nitrite values in the medium without cells were subtracted from values with cells. Control cells received drug diluent only.

*Free fatty acids (2/1 oleate/palmitate) induce damage to mtDNA in INS-1 cells. The higher the FFA concentration, the bigger is the damage. The greatest amount of damage to mtDNA was after 6 hours of incubation. FFA-induced(2mM) mtDNA damage was prevented with 0.5mM aminoguanidine.*

Cells were exposed to various concentrations of FFA (0.5-2mM) for 6 h. Cells were lysed, total DNA isolated, and quantitative Southern hybridizations were performed using a mtDNA-specific probe.

*FFA treatment of INS-1 cells produced a specific reproducible mtDNA damage pattern at the nucleotide level which is identical to one produced by NO generator PAPANOate. This pattern is strikingly different from those produced by peroxynitrite or the reactive oxygen species generator xanthine oxidase/hypoxanthine.*

Cells were treated with FFA, xanthine oxidase(50mU)+ hypoxanthine(0.5mM) for 20 min,, peroxynitrite(100 $\mu$ M) for 5 min, or PAPANOate(100mM) for 20 min, a commercially available NO generator. Control cultures were incubated with regular culture medium. After treatment, cells were lysed, and DNA was isolated and subjected to ligation-mediated PCR (LM-PCR).

Conclusion: FFAs cause a dose-dependent increase in mtDNA damage. The DNA pattern at the nucleotide was identical to the one induced by pure NO. iNOS inhibitor aminoguanidine protected these cells from mtDNA damage and diminished apoptosis.

Article 3. Kharroubi I., Ladriere L., Cardozo A. K., Dogusan Z., Cnop M., Eizirik D. L. (2004) Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: Role of nuclear factor- $\kappa$ B and endoplasmic reticulum stress. *Endocrinology* 145, 5087-5096.

Aim: To clarify if FFA-induced apoptosis is associated with the activation of NF- $\kappa$ B and the induction of iNOS, and if these FFA-mediated effects result in the induction of an ER stress response in  $\beta$ -cells.

In this study one used rat islets, FACS-purified beta-cells and INS-1E cells.

Male Wistar rats were housed and rat islets were isolated by collagenase digestion. Whole islets were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 1% BSA.

For beta-cell isolation, islets were dispersed and beta-cells purified by autofluorescence-activated cell sorting (FACS). The preparations used in the experiments contained  $95 \pm 1\%$  beta-cells. Purified beta-cells were precultured for 16 h in Ham's F-10 medium supplemented with 5% fetal bovine serum, and 1% BSA.

The insulin-producing INS-1E cells were cultured in RPMI 1640 and supplemented with 10% heat-inactivated fetal bovine serum and 1% BSA.

Oleate and palmitate were solubilised in 90% ethanol, heated to 60°C and used in a 1:100 dilution in culture medium. The control solution contained a similar dilution of ethanol.



During the experiments cells were maintained under the same culture conditions but in the absence of serum.

#### Results:

*Palmitate and oleate cause apoptosis in INS-1E cells both after 24 h and 48 h. (n=5)*

*Palmitate induced more apoptosis than the same concentration of oleate did.*

*Palmitate decrease islet cell viability only after 72 h, while oleate does not. (n=4)*

*Palmitate and oleate induce apoptosis in FACS-purified beta-cells after 72 h.*

INS-1E cells were cultured for 24 and 48 h in the presence of oleate (0.5 mM), palmitate (0.25mM and 0.5mM), IL-1-beta (30 U/ml) or TNF-alfa (1000 U/ml). Rat islets were cultured for 24, 48 or 72 h in the presence of oleate (0.5mM), palmitate (0.5mM) or IL-1-beta (50 U/ml). FACS-purified beta-cells were cultured for 72 h in the presence of oleate (0.5mM), palmitate (0.5mM) or TNF-alfa (1000U/ml).

Apoptosis determination: Islets, FACS-purified beta-cells and INS-1E cells were incubated for with the DNA binding dyes propidium iodide and Hoechst 33342. The cells were examined by inverted microscopy.

*iNOS gene expression was not induced after exposure of INS-1E or rat islets to oleate or palmitate, whereas a clear induction was observed with the positive control IL-1-beta.*

INS-1E cells were cultured for 6, 24 and 48 h with oleate(0.5mM), palmitate(0.25mM and 0.5mM) or IL-1-beta(30 U/ml). Rat islets were cultured for 24 h with oleate(0.5mM), palmitate(0.5mM) or IL-1-beta(50 U/ml).

Poly(A)+ RNA was isolated and reverse transcribed. The real-time PCR amplification reaction was done.

Standard for the gene was prepared using appropriate primers in a conventional PCR and purified for subsequent analysis.

*There was no increased accumulation of nitrite in the culture media of FFA-treated cells, whereas IL-1-beta induced a marked increase in nitrite production. Mean of 3-5 experiments.*

Same culturing conditions were used as for iNOS. The method and conditions for nitrite detection in the medium is not described in the article.

*Neither oleate nor palmitate induced NF- $\kappa$ B activation in INS-1E cells examined after 30 min, 4 h, or 12 h of FFA exposure. Specific DNA binding by nuclear NF- $\kappa$ B was observed at all time points studied in INS-1E cells exposed to TNF- $\alpha$ . NF- $\kappa$ B is a transcription factor that is activated by cytokines and leads to up-regulation of iNOS in cytokine-induced apoptosis.*

Nuclear extracts were obtained from INS-1E cells. Extracted nuclear protein (4 $\mu$ g) was preincubated for 10min at 4°C with 1 $\mu$ g of poly (dIdC) in 20  $\mu$ l medium before addition of the radiolabeled probe for the NF- $\kappa$ B consensus sequence.

*The activation of ER-stress induced effectors (GADD153/CHOP, ATF-4, XBP-1, BiP, ATF-6) was increased after exposure of INS-1 cells to palmitate (0.5mM) or oleate (0.5mM).*

GADD/CHOP, ATF-4 and BiP mRNA expression was analyzed by real-time PCR. XBP-1 was evaluated by restriction analysis after PCR amplification. ATF-6 was evaluated by transfecting INS-1 cells with luciferase test plasmids and then assaying luciferase activities.

Conclusion: FFAs activate an ER stress response via an NF- $\kappa$ B- and nitric oxide-independent mechanism.

Article 4.Maestre I, Jordan J, Calco S, Reig J. A., Cena V, Soria B, Prentki M and Roche E. (2003) Mitochondrial Dysfunction is involved in apoptosis induced by serum withdrawal and fatty acids in beta-cell line INS-1. Endocrinology 144(1), 335-345.

Aim: To gain insight into the mechanisms by which elevated concentrations of fatty acids cause  $\beta$ -cell death.

In this study INS-1 cells were grown in regular RPMI medium(11,2 mM glucose) supplemented with 10% heat treated fetal calf serum (FCS) and other standard additions.

When cells were 80% confluent, they were washed twice with Krebs-Ringer bicarbonate (KRB) buffer (ph 7.4) containing 5 mM glucose and 0.07% BSA and incubated for an additional 2 d in RPMI medium containing 5 mM glucose and 10% FCS. For the experiment, cells were washed with KRB buffer and incubated in RPMI medium containing 5 mM glucose with 10% FCS or in the absence of serum with 0.5% defatted BSA. Fatty acids, staurosporine and protease inhibitors were obtained from Sigma. The stock solutions of fatty acids bound to BSA were prepared as previously described (ref.15 in this article).

## Results:

*Cells incubated with serum are mainly quiescent in the G0 phase, with very low levels of subdiploid events (cell death) or DNA synthesis (S phase). The subdiploid population increased when serum was absent from culture medium. The addition of oleate, palmitate and staurosporine, a drug that efficiently induces apoptosis, to the serum-deprived culture medium dramatically increased the number of subdiploid events in association with a decrease in the G0 population. Mean of 3 experiments.*

Cells were incubated for 36 h in culture medium containing 5 mM glucose plus 10% FCS, or medium containing 0.5% defatted BSA with 5mM glucose, or 5 mM glucose plus 0.5 mM oleate, 0.5mM palmitate or 0.2  $\mu$ M staurosporine. Then FACS (Fluorescence-activated cell sorting) analysis was performed. DNA was estimated by measuring red fluorescence after staining with 50  $\mu$ g/ml propidium iodide for 15-30 min.

*Percentage of live cells strongly decreased under a period of 72 h in cells incubated in absence of serum and with the presence of palmitate, oleate or staurosporine, compared to cells incubated in absence of serum and without fatty acids. Mean of three independent experiments.*

The percentage of live cells was determined from the total and subdiploid dead cell populations in FACS analysis. Palmitate (0.5 mM), Oleate (0.5 mM), Staurosporine (0.2  $\mu$ M). In addition there was 0.5% BSA and 5 mM glucose in every experiment.

*DNA ladder occurred in cells incubated in the presence of palmitate, oleate and staurosporin in absence of serum. No ladder was observed in cells incubated with glucose in the presence of FCS, while some ladder was observed in absence of serum and with glucose. Cells tended also to shrink and round up in the presence of fatty acids. Fatty acids caused also chromatin condensation. All these three characteristics are typical of apoptotic cells. Results are based on three independent experiments.*

Cells were cultured for 36 h with 5mM glucose + 10% FCS, 5mM glucose + 0.5% defatted BSA without or with fatty acids. Palmitate 0.5mM, Oleate 0.5 mM, Staurosporine 0.2  $\mu$ M.

DNA ladder analysis: DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and resolved in 1.5% agarose gel containing ethidium bromide and visualized in a transilluminator.

Chromatin condensation: Cells were grown on poly-L-lysine-treated glass slides. After incubation cells were stained with 10  $\mu$ g/ml Hoechst 33342 dye for 1 min. Then samples were analyzed using a fluorescent microscope.

*Oleate and palmitate cause a rise in  $O_2^-$  production and also induce a prominent release of cytochrome c and AIF from mitochondria to the cytosol.*

Superoxide: Cells were after exposure to FFAs incubated in suspension with dihydroethidine, and superoxide production was determined by the change in fluorescence from blue to red.

Cytochrome c and AIF: Western blot were performed from the cytosolic fraction and from particulate fraction.

*Oleate and palmitate increased NO production. As positive control was used a mixture of cytokines known to be efficient in inducing iNOS and NO in this cell type. Mean of three independent experiments.*

Cells were cultured for 36 h in the presence of 0.5 mM palmitate or 0.5 mM oleate and cytokines(IL-1-beta, TNF-alfa and IFN-gamma). NO was determined as nitrite by the method of Green et al.

*Expression level of iNOS under basal conditions was below the detection limit of the Western blot and fatty acids did not appear to induce iNOS. Mean of 3 independent experiments.*

Cells were incubated for 24 h in the same test substances described above for nitrite detection. Total proteins from cells were extracted with a buffer. Western blot was performed using anti-macrophage iNOS polyclonal antibody.

Conclusion: Palmitate and oleate accelerate the apoptotic process in serum-starved  $\beta$ -cells. NO is increased apparently independently of iNOS induction. The mitochondrial arm of the apoptosis process is involved in  $\beta$ -cell lipotoxicity.

Article 5. Aarnes M., Schønberg S., Grill V. (2002) Fatty acids potentiate interleukin-1-beta toxicity in the beta-cell line INS-1E. Biochemical and Biophysical Research Communications 296, 189-193.

Aim: To investigate whether FFAs could modulate the toxicity of IL-1 $\beta$  towards  $\beta$ -cells.

In this study INS-1E cells were used. They were grown in monolayer cultures in RPMI-1640 medium containing 11 mmol/l glucose supplemented with 10% foetal calf serum and other standard additions. Cells were seeded in 75 cm<sup>2</sup> flasks at a density of 3.0x10<sup>6</sup> cells per flask. They were subcultured once a week to new flasks. The passage number of the INS-1E cells was between 61 and 75 in the present experiments.

All the fatty acids were obtained or prepared in stock ethanol solutions. Control conditions with the corresponding concentrations of ethanol (1%) were run in each experiment.

Results:

*Eicosapentaenoic acid (EPA) 70  $\mu$ M, arachidonic acid (AA) 70  $\mu$ M, linoleic acid (LA) 0.1 mM and oleic acid (OA) 0.1-0.2 mM exerted no effect on INS-1E cell viability after 2, 3,*

*and 6 days of culture. Only docosahexanoic acid (DHA) 70  $\mu$ M had a significant negative effect on cell viability after 6 days, but none after 2 or 3. Mean of 3 independent experiments.*

Cells were seeded in individual wells,  $2 \times 10^4$  cells in 100  $\mu$ l in each well in RPMI medium, and cultured for 2 days at 37°C. The medium was then replaced with test medium containing the different fatty acids described (additives). Microtiter plates containing 96 wells were cultured for 2, 3 or 6 days. Cell viability was determined by MTT assay. A stock solution of MTT (5mg/l) was added to cell-containing wells after first removing additives and medium. The plates were then incubated for 3 h. 2-propanol supplemented with HCl was added to solubilise the MTT formazan. The plates were then placed on a mechanical shaker for 20-60 min at a room temperature for complete solubilisation. Absorbency was measured on a multiscan plus reader with a 588-nm wavelength filter.

*IL-1-beta reduced the viability of cell by 44% after 2 days and by 24% after 6 days of culture. This effect of IL-1-beta was enhanced by all FFAs tested after 6 days of culture, even though EPA, AA, LA and OA by themselves failed to reduce cell viability (above).*

The same precedure as above, except for addition of 0.5 ng/ml IL-1-beta. Viability determined by MTT assay described above.

*Culture with L-NAME, a cNOS inhibitor, improved the cell survival during exposure to IL-1-beta. However, the addition of L-NAME did not abolish the potentiation effect of oleic acid on IL-1-beta toxicity.*

Same procedure as above, except for addition of 2.0 mM L-NAME

Conclusion: FFAs potentiate toxic effects of IL-1 $\beta$  on  $\beta$ -cells by mechanisms that include NO-independent ones.

Article 6. Beeharry N., Chambers J. A. and Green I. C. (2004) Fatty acid protection from palmitic acid-induced apoptosis is lost following PI3-kinase inhibition. Apoptosis 9, 599-607.

Aim: To investigate which fatty acids could induce cell death in RINm5F cell line and the contribution of iNOS to cell death signalling.

In this study RINm5F insulin-secreting cells were cultured in RPMI-1640 medium supplemented with 5% v/v heat-inactivated foetal calf serum (FCS) and other standard additions. Cells were plated at a density of  $8 \times 10^4$  (48 well plate) or  $2 \times 10^5$  cells/well (24 well plate). Stock palmitic, stearic, oleic acid were conjugated to fatty acid free bovine serum albumin (BSA) in a 3:1 molar ratio at 37°C for at least 1 h before treatment. The linoleic acid used was purchased pre-conjugated to albumin.

#### Results:

*Palmitic and stearic (saturated), but not oleic (mono-unsaturated), acid decreased RINm5F viability in a dose dependent manner. Mean of 4 independent experiments.*

Cells were exposed for 24 h to palmitic, stearic and oleic acid over a dose range (0-500  $\mu\text{mol/l}$ ). The MTS assay was used to determine cell viability based on the bioreduction of the tetrazolium compounds MTS and an electronic coupling reagent PES (phenazine ethosulfate) to produce a coloured formazan product which is proportional to the number of living cells.

*Exposure of RINm5F cells to palmitic acid caused a significant increase in apoptosis. Mean of 4 experiments.*

Cells were exposed to palmitic acid (250  $\mu\text{mol/l}$ ) for 6 and 24 h. RINm5F cell pellets were resuspended with HPI dye (Hoechst 3334 and propidium iodide, final concentrations 10  $\mu\text{g/ml}$ ). Nuclei (approximately 2000 per treatment) were scored using a fluorescent microscope. Apoptotic nuclei were distinguished as those with characteristic chromatin condensation and nuclear fragmentation.

*1400 W, a selective inhibitor for inducible nitric oxide synthase, NOS2, which inhibited nitric oxide production, had no protective effect on palmitic acid-induced cell killing. Mean of 3 independent experiments.*

Cells were exposed to palmitic acid (250  $\mu\text{mol/l}$ ) and 1400 W (200  $\mu\text{mol/l}$ ) for 24 h. Cell viability was determined by the MTS assay described above.

*Nitrite was undetectable in the medium and NOS2 protein was undetectable in Western blots of extracts of palmitate treated RINm5F cells.*

Determination of nitrite accumulation in culture medium was by the Griess assay. Western blotting for NOS2 was performed using the polyclonal antibody from Beckton Dickinson and ECL Western Blotting analysis system from Amersham Biosciences.

*It was confirmed that RINm5F cells were capable of producing NO by exposing them to cytokines (not specified which ones) and this showed an accumulation of nitrite in the medium. This nitrite generation was inhibited by 1400 W 200  $\mu\text{mol/l}$ . In addition cells exposed to IL-1-beta showed a clear expression of NOS2.*

No explanation about concentrations of cytokines/IL-1-beta or duration. Measured by Western blotting like above.

*Linoleic acid and oleate attenuate palmitate-induced cell death.*

Cells were exposed to increasing concentrations of palmitate(0-500 $\mu\text{mol/l}$ ) in combination with 1, 11.9 and 119  $\mu\text{mol/l}$  linoleic acid. Also cells were exposed to palmitate(250 $\mu\text{mol/l}$ ) with oleate (250 $\mu\text{mol/l}$ ). MTS assay was used for viability measurements.

*PI3-K inhibitors LY294002 and wortmannin abolish this attenuating effect.*

Palmitate(250 $\mu\text{mol/l}$ ), linoleic acid(11.9 $\mu\text{mol/l}$ ) and either wormannin(50nmol/l) or LY294002(10 $\mu\text{mol/l}$ ) were added to the cell culture. HPI dye was used for apoptosis measurement.

Conclusion: The growth inhibitory and apoptosis-inducing effect of saturated fatty acid palmitate on RINm5F cells is prevented by co-incubation with the polyunsaturated fatty acid linoleate, but not inhibitors of NO generation. Functional PI3-K activity is necessary to prevent palmitate-induced apoptosis.



Article 7. Welters H. J., Tadayyon M., Scarpello J. H. B., Smith S. A., Morgan N. G. (2004) Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. FEBS Letters 560, 103-108.

Aim: To investigate whether long-chain mono-unsaturates differentially regulate apoptosis induced by either lipid or non-lipid (serum withdrawal; cytokines) stimuli.

In this study the insulin-secreting beta-cell line BRIN-BD11 was cultured in RPMI 1640 medium containing 10% foetal bovine serum and other standard additions. Stearate, palmitate, myristate and octanoate were each initially dissolved in 50% ethanol by heating to 70°C. Oleate and palmitoleate were dissolved in 90% ethanol. The dissolved fatty acids were then bound to albumin by mixing with fatty acid-free bovine albumin solution at 37°C for 1 h. This mixture was added to modified RPMI 1640 medium containing 5.5 mM glucose to give final concentrations of 0.5% ethanol and 1% bovine serum albumin. Cells were treated in 6 well plates seeded the previous day with  $1 \times 10^5$  cells. Control wells received vehicle only (0.5% ethanol and 1% BSA).

Results:

*Palmitate and stearate (long-chain saturated molecules) cause a large increase in cell death while palmitoleate and oleate (equivalent unsaturated molecules) and myristate and octanoate (short-chain saturated molecules) were ineffective in inducing cell death. The cell death caused by palmitate was largely mediated by increased apoptosis.*

Cells were treated with 0.5 mM of each of the fatty acids mentioned above for 18 h. For determination of cell death, cells were collected and resuspended in trypan blue. The number of live and dead cells was counted using a haemocytometer and the percentage dead cells calculated. Apoptosis was measured by measuring

caspase activation with CaspACE assay, which measures caspase activation, and by annexin V-Cy3 staining using annexin V-Cy3 apoptosis detection kit (Sigma), which recognizes apoptotic cells by binding to phosphatidylserine present in the outer leaflet of the plasma membrane.

*Palmitoleate and oleate dramatically attenuated the cytotoxic effects of the saturated molecules. This response was dose-dependent.*

BRIN cells were exposed for 18 h to 0.5 mM palmitate in the absence or presence of palmitoleate (0.5 mM), oleate (0.5 mM), myristate (0.5 mM) or octanoate (0.5 mM). For dose-dependent results cells were treated with palmitate (0.5 mM) with increasing concentrations of palmitoleate for 18 h. Cell death was measured like described above.

*Palmitoleate attenuates the cytotoxic effects induced by serum withdrawal or by exposure to cytokines.*

Cells were cultured deprived of serum (48 h) or with cytokines IL-1 $\beta$  + IFN $\gamma$  (24 h) and with palmitoleate (0.5mM). Cell death determined like described above.

*Neither palmitate nor palmitoleate induce the production of NO from BRIN cells, while cytokines do. The cytokine-induced increase in nitrite was not changed by co-incubation with palmitoleate.*

Cells were left untreated or were exposed for 24 h to palmitate (0.5 mM), palmitoleate (0.5 mM), cytokines [IL-1-beta (2 pg/ $\mu$ l) plus IFN-gamma (1 U/ $\mu$ l)] or cytokines + palmitoleate. After this time samples of medium were removed for nitrite measurement by the Greiss assay.

*Culture with palmitate increased triacylglycerol levels inside the cells.*

Conclusion: Mono-unsaturated FFAs inhibit a distal step common to both lipid and non-lipid apoptotic pathways. This effect is not NO-mediated. Neither palmitate nor palmitoleate induce the production of NO from BRIN cells.

Article 8. Dixon G., Nolan J., McClenaghan N. H., Flatt P. R., Newsholme P. (2004) Arachidonic acid, palmitic acid and glucose are important for the

modulation of clonal pancreatic beta-cell insulin secretion, growth and functional integrity. *Clinical Science* 106, 191-199.

Aim: To investigate the effects of palmitate and arachidonic acid on  $\beta$ -cell viability,  $\beta$ -cell function, NO production and the consumption of glucose, glutamine and alanine.

Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture with 10% FCS, either 11.1 or 25mM glucose and other standard additions. The cells were cultured in 50-70 ml of tissue culture medium in T175 sterile culture flasks. Cells were subsequently seeded into 96-well plates (4x10<sup>4</sup> cells/well).

Fatty acids were initially dissolved in ethanol, but the final concentration of ethanol in cell incubations was less than 0.05%. The final concentration of FFAs was 100  $\mu$ M. The final BSA concentration derived from FCS was 0.3%.

Results:

*Palmitate inhibits the 24-h growth rate of BRIN-cells, but more so at 11 than at 25 mM glucose. Arachidonic acid stimulates the 24-h growth rate of BRIN cells at 11.1 mM glucose, but not at 25 mM glucose. Mean of 5-6 experiments.*

100  $\mu$ M of palmitate or arachidonic acid was added to the medium described above for 24 h. Cell viability was determined by the MTS colorimetric assay, which is based on the ability of viable cells, but not dead cells, to reduce MTS into a formazan product, which is soluble in tissue culture medium.

*Palmitate increases apoptotic DNA fragmentation after 24 h, but to a greater extent at 11 than at 25 mM glucose. Arachidonic acid had no effect on the level of apoptosis as determined by DNA fragmentation. Mean of 3 experiments.*

100  $\mu$ M of palmitate or arachidonic acid was added to the medium described above for 24 h. Cell pellets were resuspended in lysis buffer. DNA fragments were determined using a cell death detection ELISA kit (Roche Molecular Biochemicals).

*Addition of palmitate at 11.1 or 25 mM glucose significantly increased the rate of nitrite production. Arachidonic acid significantly decreased the production of nitrite at 11.1 mM glucose, but had no effect on the production at 25 mM glucose. Mean of 5-6 experiments.*

Deproteinized samples (150 µl) at the beginning and 24 h after culture were mixed with 75 µl of ice-cold 4,4'-diamino-di-phenylsulphone (14 mM in 2 M HCl), following the addition of 75 µl of N-(1-naphthyl)ethylenediamine (4 mM in H<sub>2</sub>O), and the mixture was incubated at room temperature for 5 min before measurements of absorbance at 550 nm.

Conclusion: Palmitate induces apoptosis in β-cells and is associated with increased NO production. Arachidonic acid stimulates β-cell growth and reduces NO production, but at high glucose concentration these effects are abolished. (The results regarding β-cell function (insulin secretion) and consumption of nutrients have not been reported because of lack of relevance to the topic of this paper.)

Article 9. Okuyama R., Fujiwara T. and Oshumi J. (2003) High glucose potentiates palmitate-induced NO-mediated cytotoxicity through generation of superoxide in clonal beta-cell HIT-T15. FEBS Letters 545, 219-223

Aim: To investigate 1) if lipoapoptosis in β-cells is enhanced by high glucose, 2) if palmitate-induced cytotoxicity is mediated by NO production and 3) if high-glucose induced superoxide overproduction plays a role in palmitate-induced cytotoxicity.

In this study the hamster beta-cell line HIT-T15 was used. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and other standard additions. Cells were inoculated into wells at 3X10<sup>4</sup>/well and cultured for several days. For experiments cells were incubated in RPMI 1640 supplemented with 1% fatty-acid-free bovine serum albumin (BSA). Palmitate was prepared by dissolving powder in ethanol:H<sub>2</sub>O (1:1).

## Results:

*Palmitate induces cytotoxicity in the hamster cell-line HIT-T15 in a dose dependent manner. The amount of cells viable after 24 h of incubation was significantly lower when compared to controls. The higher the palmitate-concentration, the lower is the cell viability. At 1,5 mM of palmitate cell viability was around 30% of that of control at 2,8 mM glucose. The results are mean of 6 replicates.*

One added various concentrations of palmitate(0mM-1,5mM) and glucose(2,8 or 12,8mM). Cell viability was measured by MTT assay: A solution of 500 µg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT). The medium was aspirated, 100 µl of MTT solution added, and incubated at 37°C for 1 h. The medium was removed and 100 µl of dimethyl sulfoxide added to lyse the cells. The absorbance at 490 nm with 650 nm reference wavelength was measured.

*Palmitate-induced cytotoxicity in HIT-T15 beta-cells was caused by cell-death due to apoptosis characterized by DNA fragmentation. Cytosolic DNA-associated histone with 0.5 mM palmitate was 1,6 of that in the absence of palmitate. Mean of 4 replicates.*

HIT-T15 cells were incubated in the absence or presence of 0,5 mM palmitate for 24 h under 2,8 mM or 12,8 mM glucose. For quantitative determination of apoptotic DNA fragmentation, cytoplasmic histone-associated DNA fragments were measured with the Cell Death Detection ELISA kit (Roche).

*Palmitate treatment significantly increased the amount of nitrate/nitrite in supernatant compared to when palmitate was absent. This is the mean of 4 replicates.*

HIT-T15 cells were incubated in the absence or presence of 0,5 mM palmitate for 24 h under 2,8 mM or 12,8 mM glucose. 5 µl of the supernatant was taken and the total amounts of nitrate and nitrite in each sample determined with the Nitrate/Nitrite Fluorometric Assay kit from Cayman.

*Palmitate-induced cytotoxicity was significantly, but not completely, inhibited by simultaneous treatment with NO synthase inhibitors L-NMMA and L-NAME, but not with iNOS inhibitor aminoguanidine. Results are mean of 6 replicates.*

HIT-T15 cells were incubated in the absence or presence of 0,5 mM palmitate for 24 h under 2,8 mM or 12,8 mM glucose. 1µl of various concentrations of L-NAME and L-NMMA was added separately to give final concentrations of 2 mM. Cell viability was measured by the MTT assay described above.

Conclusion: Palmitate-induced apoptosis is NO-mediated, but not through the induction of iNOS. (Results and conclusions for aim 1) and 3) are not relevant to the topic of the paper, and therefore have been omitted).

Article 10. Cnop M., Hannaert J. C., Hoorens A., Eizirik D. L., Pipeleers D. G. (2001) Inverse relationship between cytotoxicity of free fatty acids in pancreatic islet cells and cellular triglyceride accumulation. Diabetes 50, 1771-1777.

Aim: To examine whether prolonged exposure to FFA affects survival of isolated normal rat  $\beta$ -cells and whether the outcome is related to the occurrence of triglyceride accumulation.

In this study adult male Wistar rats were sedated and killed with CO<sub>2</sub> followed by decapitation. Pancreatic islets were isolated by collagenase digestion and dissociated into single cells in calcium-free medium containing trypsin and DNase. Single beta-cells(>90% pure) and non-beta-cells were purified by autofluorescence-activated sorting.

For the experiments cells were cultured in polylysine-coated microtiter plates in Ham's F10 medium containing 6 or 10 mmol/l glucose and 1% bovine serum albumin pretreated with charcoal.

Palmitate and oleate were added after solubilization in 95% ethanol and heated to 60°C (1:100 dilution in culture medium); control conditions contained a similar dilution of the solvent.

Results:

*Oleic acid (0.5mmol/l) exerted a minor cytotoxicity effect after 48 h, but killed 25 % of the cells after 8 days. Palmitate (0.5mmol/l) killed 54% after 48 h and virtually all cells after 8 days. Mean of 6-9 experiments.*

Cells were cultured with oleate (0.125, 0.250 and 0.5 mmol/l) and palmitate (0.125, 0.250 and 0.5mmol/l) for 2 and 8 days (1%albumin, 10 mmol/l glucose). Dead cells were determined by staining with neutral red.

*Cell death occurred both through necrosis and apoptosis. The necrotic effect was completed within 2 days. The apoptotic effect became clear only after 8 days. Mean of 5-8 experiments for 2 days, and 5-13 for 8 days.*

Cells were cultured with oleate (0.125, 0.250 and 0.5mmol/l) and palmitate (0.125, 0.250 and 0.5mmol/l) for 2 and 8 days (1% albumin, 6 mmol/l glucose). The mode of cell death was determined by Hoechst 33342-propidium iodide fluorescent assay, which distinguishes the percentage of dead cells with or without a fragmented nucleus, thus quantifying the percentage of apoptotic and necrotic cells.

*The cytotoxicity of both fatty acids was lower when higher albumin concentrations were used in the culture medium.*

Same procedure was used except for trials with variations in albumin concentrations (0.5%, 1%, 2%) and 10 mmol/l glucose.

*No FFA-induced increase in cellular NO production was noticed during 24 h culture. Addition of IL-1-beta induced a high NO production. Mean of 5-9 experiments.*

Cells were cultured in medium containing 6 mmol/l glucose and 1% albumin. They were exposed to oleate (0.25 and 0.50 mmol/l), palmitate (0.25 and 0.5 mmol/l) or IL-1-beta (30 U/ml). After 24 h medium was collected for nitrite determination using Griess reagent.

*No induction of expression of iNOS mRNA was observed after 24 h exposure of FFA, whereas a clear induction occurred after IL-1-beta treatement for 24 h. Mean of 5 experiments.*

Same culturing as for nitrite. Reverse transcriptase (RT)-PCR: Cells were harvested from the wells, RNA was extracted using Dynabeads and reverse transcribed with the GeneAmp RNA PCR Kit. Reaction products were separated in an agarose gel electrophoresis, and intensity of ethidium bromide staining was quantified by Biomax 1D Image Analysis Software (Kodak).

*The cell death was not reduced by the addition of nicotinamide, superoxide dismutase or an antioxidant mixture.*

Cells were added palmitate(0.5mM) or oleate(0.5mM), each with nicotianmide(5mM), superoxide dismutase(100µg/ml) or an antioxidant mixture(Vit. C, Vit. E acetate, dithiothreitol and glutathione).

*FFAs induce an accumulation of triglycerides in  $\beta$ -cells. This accumulation is inversely correlated to the FFA-induced  $\beta$ -cell toxicity.*

Palmitate(0.25mM), oleate(0.25mM), palmitate+oleate(each 0.125mM) or bromopalmitate(0.25mM) were added to cells. Tryglyceride accumulation was measured after 2 days with Sigma Triglyceride kit, and cell death was measured after 8 days.

Conclusion: FFAs can cause death of normal rat islet cells both by apoptosis and necrosis through an NO-independent mechanism. Oxidative stress is not the main mechanism of FFA-induced cell death. The ability to of  $\beta$ -cells to accumulate triglycerides might serve as a protective mechanism against FFA-induced apoptosis.

Article 11. Bregenholt S., Møldrup A., Blume N., Karlsen A. E., Friedrichsen B. N., Tornhave D., Knudsen L. B., Petersen J. S. (2005) The long-acting glucagon-like peptide-1 analogue, liraglutide, inhibits beta-cell apoptosis in vitro. Biochemical and Biophysical Research Communications 330, 577-584.

Aim: To investigate the potential role of GLP-1/liraglutide as a regulator of  $\beta$ -cell apoptosis.

In this study newborn Wistar rats (2-6 days of age) were killed by decapitation, and pancreata were dissected. After dissection the tissue was transferred to 15 ml test tubes containing 5 ml of 900 IU/ml Clostridium collagenase(ICN) in HBSS and hand shaken for 5-10 min until the tissue was digested. The digestion was stopped and the islets were further purified by centrifugation. The islets were cultured overnight at 37°C in RPMI-1640 containing 11mM glucose, and supplemented with 10% NCS before use. For experiments, islets were cultured in wells in RPMI 1640 supplemented with 0.5% human serum.



## Results:

*FFA and Cytokines induce apoptosis in rat islet cells. This induction was inhibited by both GLP-1 and liraglutide, a long-acting GLP-1 analogue, but to a greater extent by liraglutide. Mean of 4-5 independent experiments.*

Cytokine-mediated apoptosis was induced by incubation with a cytokine-mixture (100 U/ml recombinant rat INF-gamma, 100 U/ml TNF- $\alpha$ , and 40 U/ml IL-1- $\beta$ ). FFA-mediated apoptosis was induced by incubation with 1 mM FFA (2:1, oleate:palmitate). Islets were incubated for 10 min with GLP-1 or liraglutide prior to addition of cytokines or FFA.

Measuring of apoptosis: Following 16 h of incubation, islets were harvested and incubated with 7-aminoactinomycin D (7-AAD, a DNA-binding dye). Samples were analyzed immediately after incubation using a FACScalibur (flow cytometry).

*Exposure to either cytokines or FFA induced a significant increase in the NO production by the islet cells. Liraglutide only had a marginal effect (15%) in terms of decreasing the nitrite amount in the medium irrespective of the apoptotic-inducing regimen, making it unlikely that a direct effect of liraglutide on iNOS should account for the anti-apoptotic effect described. Mean of 3-4 experiments.*

Islets were incubated for 16 h with cytokines or FFA with or without liraglutide as described above. NO-production was measured as accumulated nitrite in the medium by mixing 100  $\mu$ l with 100  $\mu$ l Griess reagents.

Conclusion: Long-acting GLP-1 analogue, liraglutide, is a potent inhibitor of both cytokine- and FFA-induced apoptosis in  $\beta$ -cells. It is unlikely that the effect is mediated through reduction of NO, but maybe further downstream of NO-effects. It is confirmed that FFAs induce production of NO and that this correlates with increased apoptosis.

Article 12. Shimabukuro M., Ohneda M., Lee Y., Unger R. H. (1997) Role of nitric oxide in obesity-induced beta cell disease. The Journal of Clinical Investigation 100, 290-295.

Aim: To examine the possibility that loss of  $\beta$ -cells in DM2 is the result of excessive NO.

This is both an in vivo and an in vitro study. Male Wistar rats were obtained. Also obese homozygous (fa/fa) ZDF-drt rats and lean heterozygous (fa/+) ZDF littermates were bred in the laboratory.

Pancreatic islets were isolated and cultured in RPMI 1640 supplemented with 10% foetal bovine serum and 2% BSA.

FFAs used were 2 mM oleate/palmitate, 2:1. The final concentration of glucose was 8 mM.

Results in vitro:

*FFA (oleate/palmitate) induces NO production in islets from Wistar rats (200 pmol/islet), lean fa/+ rats (400 pmol/islet) and prediabetic obese fa/fa ZDF rats (900 pmol/islet) after 72 h incubation. Presence in the culture medium of nicotinamide (NIC- which prevents induction of iNOS by IL-1-beta in islets) or aminoguanidine (AG- competitive inhibitor of iNOS, but lowers iNOS expression as well) reduced the FFA-induced increase in NO from all groups. Mean of 3-5 experiments.*

Islets were cultured for 72 h in medium containing either 0 or 2 mM FFA plus either 0 or 25 mM NIC, or 0 or 0.5mM AG. Rats were 6-7 week old. Nitrite was determined by collecting 250  $\mu$ l of culture medium and incubating it with an equal amount of Griess reagent for 10 min.

*FFA dramatically increased iNOS mRNA in islets from all groups. The addition of NIC to the culture medium lowered iNOS mRNA in all groups. Mean of 3-5 experiments.*

Same incubation condition as for nitrite. The mRNA was quantified by reverse transcriptase (RT)-PCR.

Results in vivo:

*NIC and AG prevent development of diabetes in prediabetic obese ZDF rats (fa/fa) compared to untreated controls. Mean of 3-6 animals.*

ZDF rats (fa/fa) were treated for 6 weeks with daily intraperitoneal injections of 0.5g NIC or 0.4g AG/kg body weight beginning at the age of 6 week. At the end of the period blood glucose levels were measured. For NIC and AG treated rats glucose levels averaged  $7.5 \pm 0.1$  and  $6.7 \pm 0.2$  mmol/l respectively, compared with  $18.0 \pm 0.4$  mmol/l in untreated controls.

*The profound reduction in the number of beta-cells in ZDF rats (fa/fa) was prevented by both NIC and AG therapy.*

Immunohistochemistry: Bouin-fixed paraffin-embedded serial sections of perfused pancreata (5- $\mu$ m thickness) were stained for insulin and GLUT-2 by indirect immunofluorescence.

*iNOS mRNA could not be detected in islets in neither (fa/+) or (fa/fa) 6-wk-old rats; iNOS mRNA was measurable in both homozygous and heterozygous 12-wk-old groups, but was 20 times higher in the homozygous rats. In 12-wk-old ZDF rats in which the diabetes had been prevented by 6 wk with NIC and AG treatment, iNOS mRNA was reduced to almost normal. Mean of 3 experiments.*

Islet freshly isolated from 6- and 12-wk-old obese fa/fa ZDF and lean fa/+ ZDF rats were semiquantified for iNOS by RT-PCR.

Conclusion: Palmitate/oleate induce iNOS expression and NO production in rat islets. NO-lowering agents prevent adipogenic diabetes and  $\beta$ -cell loss in obese rats.

Article 13. Shimabukuro M., Zhou Y., Levi M., Unger R. H. (1998) Fatty acid-induced beta-cell apoptosis: A link between obesity and diabetes. *Proc. Natl. Acad. Sci. USA* 95, 2498-2502.

Aim: To test if  $\beta$ -cell lipotoxicity in DM2 involves ceramide- and/or NO-mediated apoptosis.

This is both an in vivo and in vitro study. Lean wild-type (+/+) male ZDF rats and homozygous (fa/fa) male ZDF rats were bred in the laboratory from [ZDF/Drt-fa (F10)] rats. Pancreatic islets were isolated by the method of Naber et al. (no other explanation how in the article). Isolated islets were cultured in RPMI 1640 supplemented with 10% foetal bovine serum and 2% BSA. FFAs used in the in vitro experiments were 1 mM of (oleate/palmitate, 2:1).

Results in vivo:

*There was a more than 7-fold increase in DNA ladder formation, an index of apoptosis, in freshly isolated islets from 5-wk (early prediabetic stage), 7-wk (late prediabetic stage) and 14-wk-old (4 weeks after onset of diabetes) ZDF fa/fa rats, whereas none was detected in lean-wild-type controls. Apoptosis increased with age almost 3-fold in the obese rats. Mean of 3-4 experiments.*

Islets were isolated from obese fa/fa ZDF rats and from lean wild type +/+ ZDF rats of 5-, 7-, and 14-weeks of age. DNA fragmentation: Islets were suspended in 100 µl of lysis buffer. After centrifugation for 20 min, the supernatant containing fragmented (soluble) DNA was transferred to another tube. Samples were treated with RNase A for 1 h and then with proteinase K for 1 h. DNA concentrations were measured the next day. The soluble fraction of DNA was determined by electrophoresis on 1.5% agarose gel.

Results in vitro:

*FFAs cause DNA fragmentation in islets from both lean wild-type (from 0.7% to 4.6%) and obese prediabetic homozygous (from 6% to 19%) ZDF rats after 24 h of incubation.*

Islets were isolated from 7-wk-old rats and cultured with 1 mM FFA (oleate/palmitate, 2:1) for 24 h. DNA fragmentation was determined as described, and expressed in % of total DNA.

*DNA fragmentation was lowered substantially in islets from obese prediabetic homozygous ZDF rats incubated with aminoguanidine in addition to FFAs.*

Islets were isolated from 7-wk-old rats and cultured with 0 mM or 1 mM FFA (oleate/palmitate, 2:1) with 0.5 mM aminoguanidine for 24 h. DNA fragmentation was determined as described, and expressed in % of total DNA.

*FFAs induce iNOS mRNA expression and NO production in islets from obese fa/fa ZDF rats. iNOS expression and NO production were decreased in the islets incubated with aminoguanidine in addition to FFAs.*

Islets were isolated and cultured with 0 mM or 1mM FFA with or without 0.5 mM aminoguanidine for 24 h (iNOS mRNA determination) and 48 h (Nitrite determination). iNOS mRNA was semiquantified by reverse transcriptase-PCR, as in article 12. Nitrite was determined in the same way as in article 12.

Conclusion: In ZDF obesity,  $\beta$ -cell apoptosis is induced by increased FFA via increased NO-production and de novo ceramide formation. (Results about ceramide are not presented).

Article 14. Lupi R., Dotta F., Marselli L., Guerra S. D., Masini M., Santangelo C., Patane G., Boggi U., Piro S., Anello M., Bergamini E., Mosca F., Di Mario U., Del Prato S., Marchetti P. (2002) Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets. Diabetes 51, 1437-1442.

Aim: To assess whether prolonged exposure to FFAs affects the survival of isolated human islet cells and, if so, which pathways are involved.

Pancreatic islets were obtained from 18 non-obese human multiorgan donors (10 men and 8 women, aged  $63 \pm 10$  years, BMI  $24 \pm 2$  kg/m<sup>2</sup>); cause of death: 11 cerebral vascular event and 7 trauma).

The pancreata were digested with collagenase and islets were isolated. They were resuspended in M199 culture medium, supplemented with 10% adult bovine serum and

antibiotics and cultured. For experiments the culture medium consisted of the supplemented M199 medium, either with or without 1.0 or 2.0 mmol/l (2:1, oleate/palmitate) containing 2% human albumin.

## Results:

*FFAs induce an increase in the amount of dead cells (in reality it's DNA fragmentation that is increased if you look at the methods used below, but the writers conclude directly as if it is cell death) in human islets compared to control. TUNEL-results are from 4 separate pancreata, ELISA-results are from 9 separate pancreata.*

Islets were exposed for 48 h to 2.0 mmol/l FFA-mixture.

Islet death was assessed by TUNEL (transferase mediated dUTP nick-end labelling)-technique, and by ELISA plus assay (Roche) with anti-histone-biotin antibody and anti-DNA-peroxidase antibody.

*Beta-cells were involved in this cell death phenomenon described above. In addition they showed morphology suggestive of apoptotic phenomena.*

Aliquots of control and FFA exposed islets were sampled for electron microscopy analysis. Beta-cells, recognized for their insulin granules, showed changes suggestive of apoptotic phenomena: autophagy and chromatin condensation.

*Inhibition of NO synthesis by the use of n-NAME did not affect the rate of human islet cell death, whereas VAD-FMK (inhibitor of upstream caspases), DCI (inhibitor of serine proteases) and myriocin (inhibitor of ceramide synthesis) reduced islet cell death.*

Islets were exposed for 48 h to 0 or 2.0 mmol/l FFA-mixture with or without n-NAME (10 mmol/l), VAD-FMK, DCI or myriocin. Cell death was assessed by the ELISA technique described above.

*iNOS mRNA expression was not changed by FFAs, while Bcl-2 mRNA expression was markedly reduced.*

Islets were exposed for 48 h to 0 or 2.0 mmol/l FFA-mixture. Reverse-Transcriptase-PCR was performed in order to analyze mRNA expression of iNOS and Bcl-2.

*Triglyceride content increased in islets exposed to FFAs for 48 h.*

Conclusion: Prolonged exposure to FFAs has pro-apoptotic effects on human pancreatic  $\beta$ -cells, and the effects are mostly caspase mediated, partially dependent on ceramide pathway, and possibly Bcl-2 regulated. NO does not seem to mediate these effects.

Article 15. Ekelund M., Qader S. S., Jimenez-Feltstrom J., Salehi A. (2006)  
Selective induction of inducible nitric oxide synthase in pancreatic islet of rat after an intravenous glucose or intralipid challenge. *Nutrition* 22, 652-660.

Aim: To investigate the effect of short-term (24 h) infusion of glucose or Intralipid on the expression of iNOS in pancreatic islets.

This is an in vivo study using male Sprague-Dawley rats weighing 200 to 225 g. The rats intended for i.v. nutrient administration were intraperitoneally anesthetized with 5% chloral hydrate before operation. The neck of the rat was shaved and a catheter was inserted into the right external jugular vein. The rats serving as freely fed controls or those orally fed underwent the same procedure. At the end of experiment (24 h later), the catheter was flushed with low-molecular-weight heparin before aspiration of blood.

The rats were assigned to one of four groups: freely fed (FF)-free access to a standard pellet diet and tap water ad libitum, oral glucose (OG)-provided with batches of 6 ml of glucose (50%) each third hour, IV glucose (IVG)-received a continuous IV infusion (2 ml/h) of a 50% glucose solution, or IV Intralipid (IL)-received a continuous IV infusion (2 ml/h) of Intralipid. Preparation of isolated pancreatic islets from rat was performed by retrograde injection of a collagenase solution through the bile-pancreatic duct. Islets were then collected under stereomicroscope at room temperature.

Results:

*Islets cells isolated from IVG and IL showed a great immunoreactivity for iNOS, whereas islets from FF and OG showed no immunoreactivity. Expression of iNOS in IVG and IL was confirmed by Western blot analysis, while no expression was showed in FF and OG. Islet ncNOS expression was decreased in IL group. Mean of 5 experiments.*

Immunocytochemistry: Freshly isolated islets were fixed and permeabilized. iNOS were detected with corresponding rabbit-raised primary antibodies in combination with Cy2-conjugated anti-rabbit IgG. Fluorescence was visualized.

Western blot: Immunoblotting with rabbit anti-mouse iNOS or ncNOS was performed.

*There was a strong upregulation of islet iNOS activity in IVG and IL groups. No iNOS activity was detected in islets form FF and OG groups. ncNOS activity in the islets if IL was slightly decreased. Total NOS activity was clearly increased in IVG and IL groups.*

Islets were washed and collected in ice-cold buffer, supplemented to contain CaCl<sub>2</sub>, calmodulin, NADPH and L-arginine. For the assay of iNOS calmodulin and CaCl<sub>2</sub> were omitted from the buffer. The homogenate was then incubated under constant air bubbling for 3 h. The amount of L-citrulline formed (NO and L-citrulline were produced in equimolar concentrations) was measured.

Conclusion: The impaired  $\beta$ -cell function found after 24 h of i.v. infusion of glucose or Intralipid might be mediated, at least in part, by the induction of iNOS in pancreatic islets.



## Discussion

During the last decade there has been a lot of research on the topic of DM2. A lot of different terms and phenomena have evolved as a result of this; lipotoxicity, glucotoxicity,  $\beta$ -cell dysfunction and  $\beta$ -cell death to mention a few (6). For each of these topics a lot of different theories have evolved regarding the mechanisms of how they happen. This paper has one of the theories of FFA-induced apoptosis; NO-mediated apoptosis, as the main topic. There has been a lot of contradicting publications regarding this theory; some supporting, while others opposing it. This paper has made an effort to review supporting and opposing articles, and try to make a conclusion.

In the “insulinitis” in DM1, cytokines up- or downregulate  $\approx 700$  genes in  $\beta$ -cells (5). IL-1 $\beta$  activates the transcription factor NF- $\kappa$ B in rodent and human islet cells. This factor regulates  $>60$  genes, some of them being transcription factors themselves, thus amplifying its effect. NF- $\kappa$ B regulates expression of iNOS in  $\beta$ -cells, and  $\approx 50\%$  of the  $\beta$ -cell genes modified after exposure to cytokines are secondary to iNOS-mediated NO formation (8). Among these are p38 MAPK, ERK and p53, which are pro-apoptotic. Also NO disrupts ER homeostasis by depleting Ca $^{2+}$  stores, inhibiting SERCA2b expression and then activating diverse components of the ER stress response (5). As we can see NO is a very important mediator of cytokine-induced apoptosis.

DM2 is not an autoimmune disease. The risk factors like central obesity, physical inactivity and saturated fat-rich meals are well known. Autopsy data suggest that the progressive decline in insulin secretion in DM2 is accompanied by a decrease in  $\beta$ -cell mass and that this is secondary to increased  $\beta$ -cell apoptosis. And it has been established that high plasma [FFA] is a risk factor for the development of DM2 (9). This links FFA to apoptosis in vivo, but apart from this most evidence for FFA-induced apoptosis is derived from in vitro experiments. Because of NOs role in DM1 one has tried to investigate its role in DM2.

Now let us turn towards the 15 articles presented in this paper, which include 5 in vitro studies on INS-1 cells, 5 in vitro studies on other  $\beta$ -cells, 5 in vitro studies on pancreatic islets and 3 in vivo studies on rats. This is a lot of information, so the comparable and most important information has been systematized in different tables. When one investigates NO's role in apoptosis, there are a lot of parameters one needs to take into account. Some of the important ones are: NO detection, iNOS expression/activity, NF-kB expression, apoptosis, effects of inhibiting NO production and other typical NO damages (ER stress,  $O_2^-$  etc). Apart from this, one needs to consider methods by which results have been obtained, and if they are satisfactory.

### **Methodological considerations**

Methods for measuring the different parameters: Every one of the 15 articles uses methods that are generally accepted as satisfactory. Griess reaction for NO, Western blot for detection of proteins, RT-PCR for mRNA, MTT, MTS, ELISA, HPI dye, trypan blue, neutral red or DAPI are used for viability, DNA fragmentation, apoptosis and cell death in general. All of these have been bought from similar companies and used according to manufacturers' instructions.

### **Independent experiments:**

NO detection:     $\uparrow$ : 1(n=3), 2(n=x), 4(n=3), 8(n=5-6), 9(n=4), 11(n=3-4), 12(n=3-5), 13(n=x).  
                           $\leftrightarrow$ : 3(n=x), 6(n=x), 7(n=x), 10(n=5-9).

iNOS detection:     $\uparrow$  : 1(n=3), 12(n=3-5), 13(n=x), in vivo: 12(n=3), 15(n=5)  
                           $\leftrightarrow$ : 3(n=3-5), 4(n=3), 6(n=x), 10(n=5), 14(n=x)

As we can see most of the articles have 3-6 independent experiments, which give them credibility for the results. Quality-wise they are therefore comparable.

Approaches: All the in vitro studies had the same approach: culturing of cells in very similar conditions, usually RPMI 1640 with 10 FBS. And then they exposed the cells without FBS,

but instead BSA, to different additives (FFA, A, NIC, NAME etc.) Only in article 4, 6 and 7 was the FFA solution pre-conjugated with BSA before additions to cells. In the rest of them BSA was already in the media when the FFA solution was introduced. This is of importance, because the not-conjugated ones will have a much higher [FFA] on the cell membrane and lead to higher flux into the cells. But because almost all of the articles didn't pre-conjugate, this makes them comparable to each other.

[FFA] and relation to BSA: This relationship and studies where [FFA]>0.5mM, because of higher risk of unspecific cell death, will be discussed individually under review below.

## **Review**

Article 1: Ol/P, 2:1 (2.0mM) was used on INS-1 cells. Increased DNA fragmentation, mtDNA damage, NO production, iNOS induction and decreased viability was established. Transfected MTS-OGG1 cells showed a large decrease in DNA fragmentation, mtDNA damage and large increase in viability.

Unspecific cell death? I believe it is not because: 1) Mitochondria damage is the pathway of apoptosis here; in unspecific death it would be more evenly distributed to other organelles: nucleus (DNA), cell membrane etc. 2) Unspecific death would be dominated by necrosis, which is not the case here.

NO and iNOS increase show a correlation with FFA-induced apoptosis, but not a causative direct link. To attain this link one needs to show that NO-inhibitors attenuate apoptosis.

Article 2: FFAs(2mM) induce apoptosis, which is strongly attenuated by aminoguanidine. There is increased NO production, which is blocked by aminoguanidine. Also there is increased mtDNA damage, which is prevented by aminoguanidine. The mtDNA damage pattern is identical to the one produced by pure NO (PAPANOate). Unspecific cell death?

The answer is no because: 1) Apoptosis and mtDNA damage is prevented with iNOS inhibitor. 2) The mtDNA damage pattern is identical to pure NO.

Article 3: Palmitate and oleate (both 0.5mM) induce apoptosis in INS-1 and FACS-purified  $\beta$ -cells and reduce viability in Wistar rat islets. There is no increase in NO production or iNOS expression in INS-1 cells or islets and no induction of NF-kB in INS-1 cells, while cytokines induced all of them. There is activation of ER stress-induced effectors. This argues against NO as mediator, and shows that ER stress occurs independent of NO.

Article 4: Palmitate and oleate (both 0.5mM) increase cell death, apoptosis and decrease viability in INS-1 cells. They increase NO-production, but not iNOS. Also they cause a rise in  $O_2^-$  production and also induce a prominent release of cytochrome c and AIF from mitochondria to the cytosol. This shows that the mitochondria are involved in FFA-induced apoptosis and this is correlated with NO, but not through iNOS. Again this is just a correlation and not a direct causative link.

Article 5: Oleate (0.1, 0.2mM) and Linoleic acid (0.1mM) do not reduce viability in INS-1 cells, but they potentiate IL-1 $\beta$ -induced cytotoxicity. This potentiation is not inhibited with cNOS inhibitor. The authors argue based on this that the potentiation is not NO-mediated. I don't agree, because they also should have used an iNOS inhibitor to exclude the possibility of iNOS induction.

Article 6: Palmitate and stearic acid (both 0.0-0.5mM), but not oleate, reduce viability in RINm5F  $\beta$ -cells. This is not inhibited by iNOS inhibitor. Palmitate also induces apoptosis, and there is no increase in NO or iNOS production. Cytokines induced NO and iNOS in this cell type. Linoleic acid and oleate attenuated palmitate-induced cell death, and this seems to be PI3-K mediated. This argues against NO as mediator, but they should have tested with cNOS inhibitor as well.

Article 7: Palmitate and stearate (both 0.5mM) induce cell death in BRIN cells, while palmitoleate and oleate(both 0.5mM) don't. The cell death is through apoptosis. Palmitoleate attenuated the cytotoxic effects of palmitate, serum-withdrawal and cytokines. Neither palmitate nor palmitoleate increased NO production. The only argument here is that there was no NO production, this is again just a correlation.

Article 8: Palmitate (0.1mM) reduces viability, increases apoptosis and NO production in BRIN cells. Arachidonic acid (0.1mM) increases viability and decreases NO production. Again here is a correlation between apoptosis and NO. Also shown is a possible mechanism, that viability can be increased by reducing NO amount.

Article 9: Palmitate(0.0-1.5mM) reduce viability in hamster  $\beta$ -cells HIT-T15, and this is mainly through apoptosis. NO production was increased, and viability reduction was attenuated with cNOS inhibitors, but not with iNOS inhibitor. This shows a direct link between increased NO and decreased viability, but not through iNOS.

Article 10: Oleate and palmitate (both 0.5mM) induce cell death in FACS-purified  $\beta$ -cells from Wistar rats. Death was both through apoptosis and necrosis. There was no increase in NO or iNOS, while cytokines induced both. No inhibition of cell death was achieved with iNOS inhibitor, anti-oxidative compounds and free radical scavengers. The triglyceride accumulation in the cells was inversely correlated to FFA-induced  $\beta$ -cell toxicity. This argues strongly against NO as mediator in FFA-induced apoptosis.

Article 11: Oleate/palmitate, 2:1 (1mM) induces apoptosis in Wistar rat islets. NO production is also increased. Lirglutide inhibits both cytokine- and FFA-induced apoptosis, but not through reduction of NO. Inhibitor of PI3-K abolished this inhibitory effect. Again the only argument here is correlation NO and apoptosis. Unspecific cell death? I believe it is not, because of the specific blocking effect of wortmanin, PI3-K inhibitor.

Article 12: Oleate/palmitate, 2:1 (2.0 mM) increase NO production and induce iNOS in islets from Wistar, ZDF(fa/+) and ZDF(fa/fa) rats. NO production and iNOS induction are inhibited with iNOS inhibitors in all islets. In vivo: Treatment with iNOS inhibitors from 6 to 12 weeks of age prevents development of DM and  $\beta$ -cell loss, and decreases expression of iNOS in ZDF(fa/fa) rats. No cell death has been tested for in the in vitro part, but [FFA] is high. Can unspecific cell damage cause increased NO and iNOS? NO can be toxic in a number of different cells, but the fact that we see an induction of iNOS and a reversion with iNOS inhibitors argues against that this is unspecific cell damage. The in vivo experiment is a very strong argument for NO as mediator of FFA-induced apoptosis.

Article 13: ZDF(fa/fa) rats (5,7,14 weeks) have a 7-fold increase in DNA fragmentation compared to ZDF(+/+). In vitro: Oleate/palmitate, 2:1 (1 mM) cause DNA fragmentation in ZDF(+/+) and ZDF(fa/fa) rats. Also they increase NO production and induce iNOS.

Fragmentation, NO production and iNOS induction is inhibited with iNOS inhibitors.

Unspecific cell death? There is no unspecific death because of the inhibition with iNOS inhibitor. This shows a direct causative link between apoptosis and increased iNOS and NO.

Article 14: Oleate/palmitate, 2:1 (2.0 mM) induce cell death through apoptosis in human islets.  $\beta$ -cells were involved in this cell death effect. cNOS inhibitor did not inhibit cell death, whereas VAD-FMK (inhibitor of upstream caspases), DCI (inhibitor of serine proteases) and myriocin (inhibitor of ceramide synthesis) reduced islet cell death. iNOS was not induced, while expression of Bcl-2 was reduced. Unspecific cell death? Because of inhibition of different specific pathways, it doesn't seem that there is unspecific cell death. This article suggests that classical caspase proteases, noncaspase proteases and ceramide formation have a role in FFA-induced cell death, but that it is not NO mediated.

Article 15: Intravenous infusion of lipids induces iNOS in islets of Sprague-Dawley rats.

Islets are capable of inducing iNOS in vivo in response to short time (24 h) exposure to lipids.

## **Sum up and Conclusion**

We start with the issue of higher risk of unspecific cell death with [FFA]>0.5mM. Article 1, 2, 11, 12, 13 and 14 employ [FFA]>0.5mM in their experiments. As discussed for each of the articles in the review above, I don't think that unspecific cell death has occurred because: Art. 1) Apoptosis occurs through mtDNA damage; in unspecific death it would be more evenly distributed to other organelles: nucleus (DNA), cell membrane etc. Unspecific death would be dominated by necrosis, which is not the case here. Art. 2) Apoptosis and mtDNA damage is prevented with iNOS inhibitor and the mtDNA damage pattern is identical to pure NO. Art. 11) There is a specific blocking effect of wortmanin (PI3-K inhibitor). Art. 12) We see an induction of iNOS and a reversion with iNOS inhibitors. Art. 13) Apoptosis is inhibited with iNOS inhibitor. Art. 14) Apoptosis is inhibited by inhibition of different specific pathways (ceramide synthesis, serine proteases, upstream caspases)

What can we conclude from these articles?

All of the experiments in articles 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13 and 14 that tested if FFAs induced apoptosis in  $\beta$ -cells or islets, showed that they actually did. Not only in vitro, but also article 13 shows that apoptosis is induced in pre-diabetic and diabetic obese rats in vivo, and that it increases with age/as diabetes develops. This confirms that the phenomenon lipotoxicity does exist.

However, not all fatty acids promote similar reactions. Article 6(RIN-m5F) and 7(BRIN-BD11) report that unsaturated fatty acids like oleate, linoleic acid and palmitoleate did not induce cell death or decreased viability, while saturated palmitate and stearic acid did. Actually they even attenuated the cytotoxic effect of saturated FFAs. Also arachidonic acid wasn't toxic to BRIN-BD11 cells (Art. 8), but rather growth stimulating. In INS-1 cells and FACS-purified  $\beta$ -cells oleate induced cell death, but less than palmitate. This is in agreement

with other reports showing that the degree of the saturation of fatty acid dictates the extent of cell death (25, 26).

Another issue that these articles agree on is the involvement of mitochondria in FFA-induced apoptosis in INS-1 cells. Article 1 and 2 show that FFAs induce apoptosis via damage of mtDNA, and that apoptosis is reduced by transfecting and targeting DNA repair enzyme hOGG1 to mtDNA. Also in article 4 FFA-induced mitochondrial damage is demonstrated by a rise in  $O_2^-$ , and release of cytochrom c and AIF from mitochondria to cytosol.

Triglyceride formation in cells exposed to FFAs is shown in articles 8, 10, 13 and 14. However, article 10 shows an inverse relationship between triglyceride amount and cytotoxicity, suggesting that TAG-formation may be cytoprotective. What the role of triglyceride accumulation in  $\beta$ -cells is unknown. Some claim that it is beneficial to  $\beta$ -cell survival (19, 27), while others claim it is causative (28, 29).

#### NO's role:

Supports: It is shown a mtDNA damage pattern in INS-1 (Art. 2) that is identical to the one from pure NO. This mtDNA damage and the FFA-induced apoptosis are inhibited with iNOS inhibitor aminoguanidine. FFA-induced reduction in viability in hamster HIT-T15 cells (Art. 9) is attenuated with cNOS inhibitors NAME and NMMA, but not with aminoguanidine. In article 12 treating ZDF(fa/fa) rats with iNOS inhibitors nicotinamide or aminoguanidine subperitoneally for 6 weeks prevented development of diabetes and loss of  $\beta$ -cells, and decreased iNOS expression in islets compared to non-treated controls. In article 13 aminoguanidine attenuated the apoptotic effects of FFAs on ZDF rat islets. In 2(INS-1) and 9(HIT-T15) there was increased NO production, which was inhibited by aminoguanidine and NAME/NMMA respectively. In 12(Wistar and ZDF) and 13(ZDF) islets increased nitrite and iNOS, and both were inhibited with aminoguanidine. In article 8(BRIN-BD11) there is a



correlation between arachidonic acids  $\beta$ -cell growth stimulatory effect and decrease in NO, which suggests that NO reduction may be growth-stimulatory. And article 15 demonstrates that in vivo i.v. infusion of lipid to normal rats induces iNOS expression and activity. Also article 1(INS-1), 4(INS-1), 8(BRIN-BD11), 11(Wistar islets) show positive correlations between FFA-induced apoptosis and increased NO production.

Opposes: FFAs induce ER stress response in INS-1 cells (Art. 3), but no NO, iNOS or NF- $\kappa$ B induction is observed. In  $\beta$ -cells from Wistar rats (Art. 10) there was no increase in NO or iNOS, and nicotinamide, anti-oxidative compounds and free radical scavengers had no effect on cell viability. One of NO's ways to damage cells is through generation of peroxynitrite, a reactive oxidant (18). The fact that anti-oxidative compounds and free radical scavengers had no effect on viability is considered, according to the authors, as an argument against NO damage. But this is not the only way NO can damage, in fact it is demonstrated in article 2 that NO damage occurred through formation of N<sub>2</sub>O<sub>3</sub> (nitrous anhydride), which has the ability to deaminate bases(11). 1400W, an iNOS inhibitor, had no effect on the reduction of viability caused by palmitate in RIN-m5F cells, and there was no NO or iNOS induction (Art 6). In the only human islet study (Art. 14) there was not shown iNOS induction, and NAME had no effect on cell viability. Indeed it seems that caspases, serine proteases, ceramide synthesis and reduction of Bcl-2 play a role in FFA-induced apoptosis in human islets. Also there was no increase in NO in article 7.

As we can see some of the results oppose each other. As discussed under methodological considerations, the results are valid and none of the articles can be considered better than the others in terms of methodological quality.

Why is it then that some cells produce NO, iNOS and react to iNOS/cNOS inhibitors, while others don't? This is a difficult question. There is nothing in the articles who oppose the hypothesis that differentiate them from others that support it; Cells used are the same, fatty

acid concentrations and BSA% are similar, methods for the detection of results are the same as in the ones supporting the hypothesis. This leads to speculations, and one possible explanation is that cells, like any other living organisms, are not the same. During one culturing process cell go through a lot of replications, they change their properties and the researches end up working on cells that are different. So then some cells may react to FFAs with NO production that leads to damage, while others initiate damage in other ways.

### **Conclusion:**

Because 1) the only in vivo study strongly supports NO-mediated apoptosis, and shows powerfully how diabetes can be prevented with iNOS inhibitors, 2) numerous in vitro studies show attenuating effects on apoptosis by iNOS inhibitors, and one of them by cNOS inhibitor 3) ER stress does not correlate with NO/NF-kB only is a negative correlation and not a direct link, 4) and most importantly because positive results weigh more than negative, my conclusion is that NO may play a role as a mediator in FFA-induced apoptosis in rodent  $\beta$ -cells.

How NO is induced and how it imposes damage is probably somehow different from cytokine-induced NO. This is because 1)NF-kB does not seem to be activated by FFAs, 2)it has been observed that cNOS inhibitors attenuate apoptosis, 3)NO production can be increased without induction of iNOS, and 4)NO has been shown to damage mtDNA directly through N<sub>2</sub>O<sub>3</sub>, and not through regulation of different genes like in cytokine-mediated apoptosis.

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