Buněčná a molekulární fyziologie lipidů

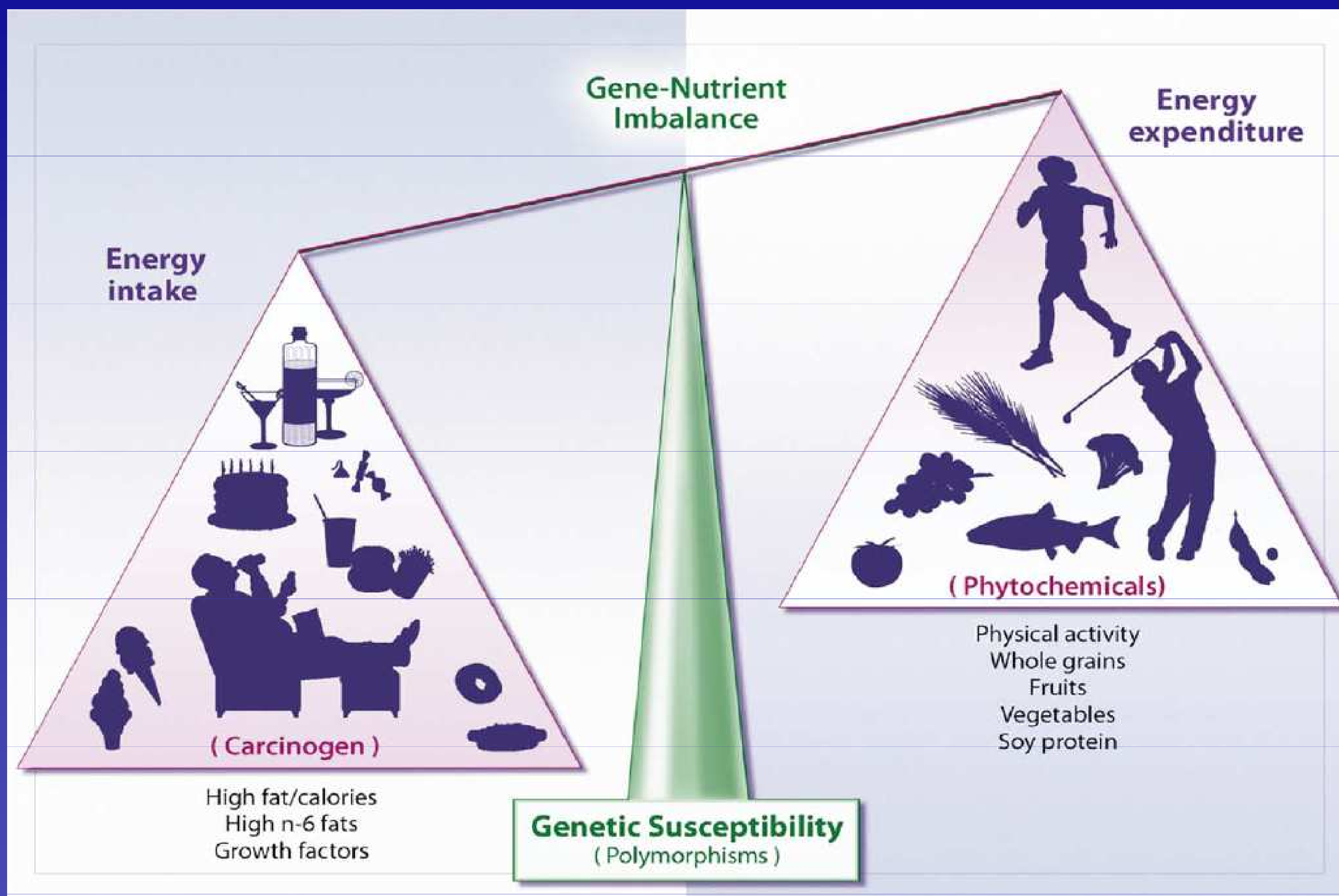
Doc. Jiřina Hofmanová

Změny cytokinetiky epiteliálních buněk kolonu indukované nutričními faktory *in vitro*

Interakce endogenních regulátorů a lipidových složek výživy, molekulární mechanizmy

Laboratoř Cytokinetiky
Biofyzikální ústav AVČR, BRNO

Nerovnováha mezi příjmem a výdejem energie ve vztahu k obezitě a chronickým onemocněním



Genetické polymorfismy posouvají rovnováhu mezi příjmem a výdejem energie. Vyšší příjem energie, nízký poměr nenasycené/nasycené tuky, inzulinová resistence, a sedavý životní styl jsou rizikovými faktory vedoucími k obezitě a chronickým onemocněním (srdeční choroby, diabetes a nádory). To je vyvažováno fyz. aktivitou a dietou bohatou na celá zrna, ovoce, zeleninu, soju atd., která snižuje riziko těchto chorob. (Trujillo E., J Amer Diet Assoc., 106, 2006)

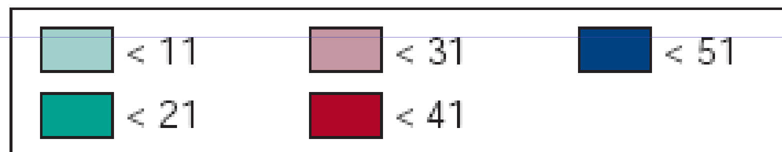
PŘÍČINY VZNIKU KARDIOVASKULÁRNÍCH A NÁDOROVÝCH ONEMOCNĚNÍ GENETICKÉ +FAKTORY ŽIVOTNÍHO STYLU

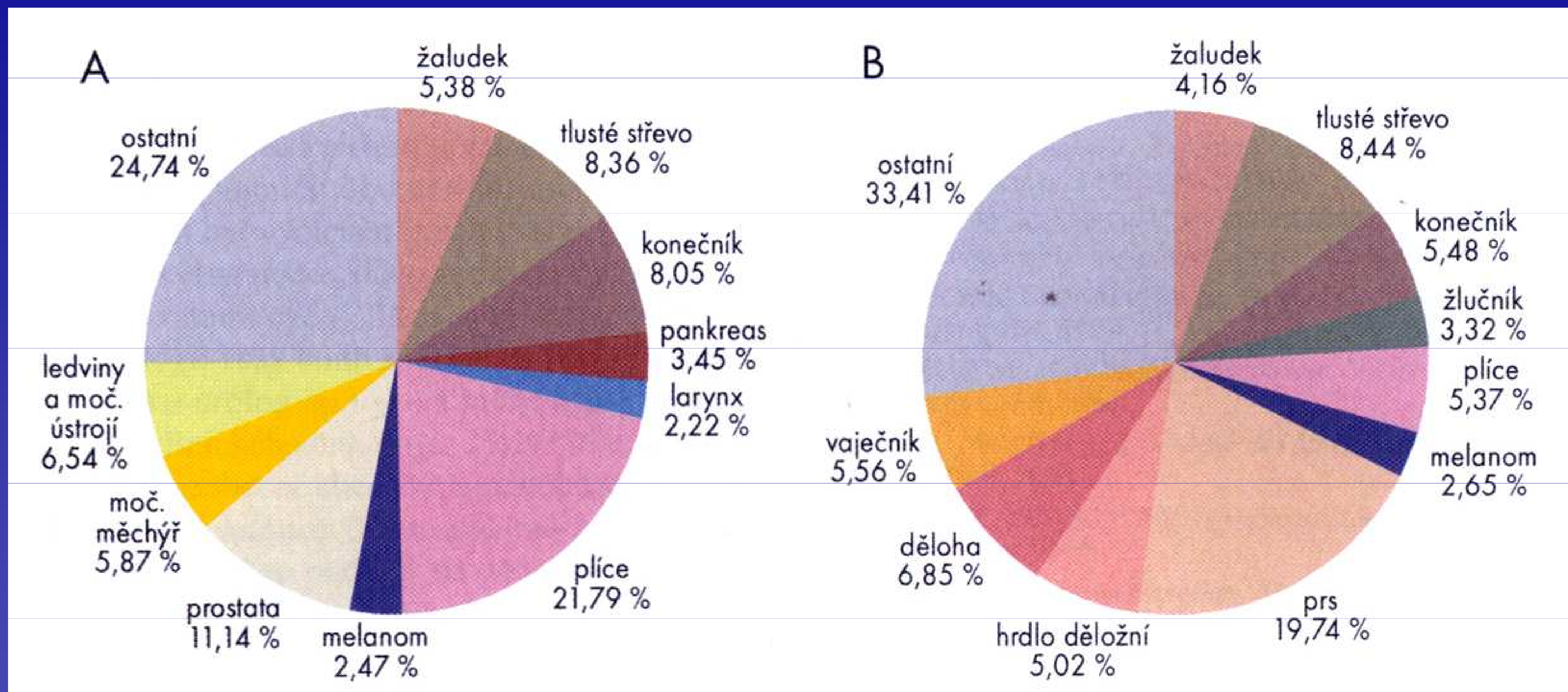
TABÁK a VÝŽIVA (podílejí se až na 2/3 úmrtí a jsou nejvíc ovlivnitelné)
Tučná jídla (zvýšené riziko), ovoce a zelenina (prevence)

Strategie minimalizace rizika vzniku onemocnění

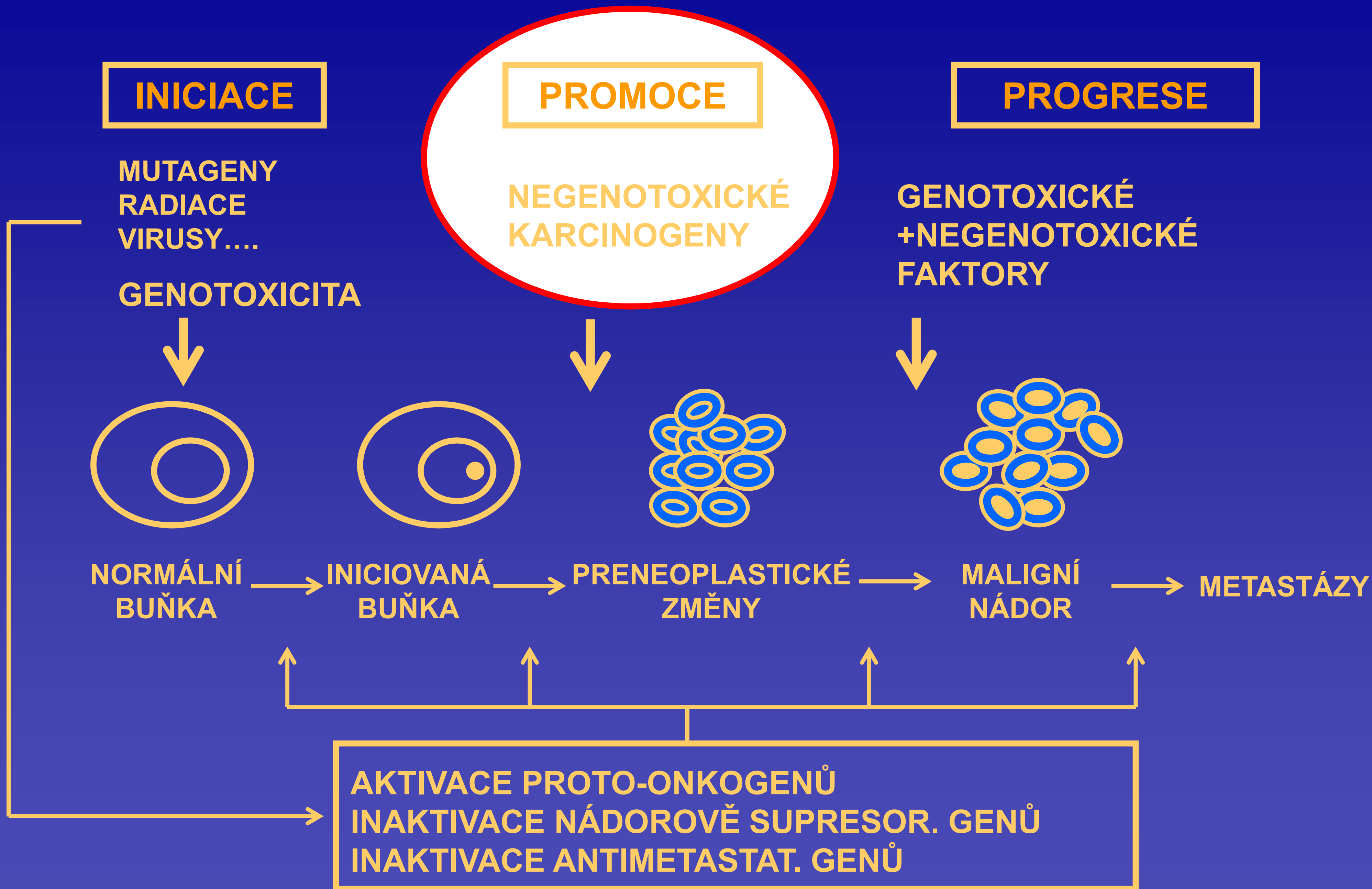
FYZICKÁ AKTIVITA, RELAXACE, „MÍR NA DUŠI“,
OMEZENÍ STRESU,
ŽÁDNÉ KOUŘENÍ, SPRÁVNÁ VÝŽIVA

a Incidence rates of colorectal cancer





Struktura hlášených onemocnění novotvary bez dg. C44. A – muži; B – ženy (podle ÚZIS)



Mnohostupňový proces karcinogeneze

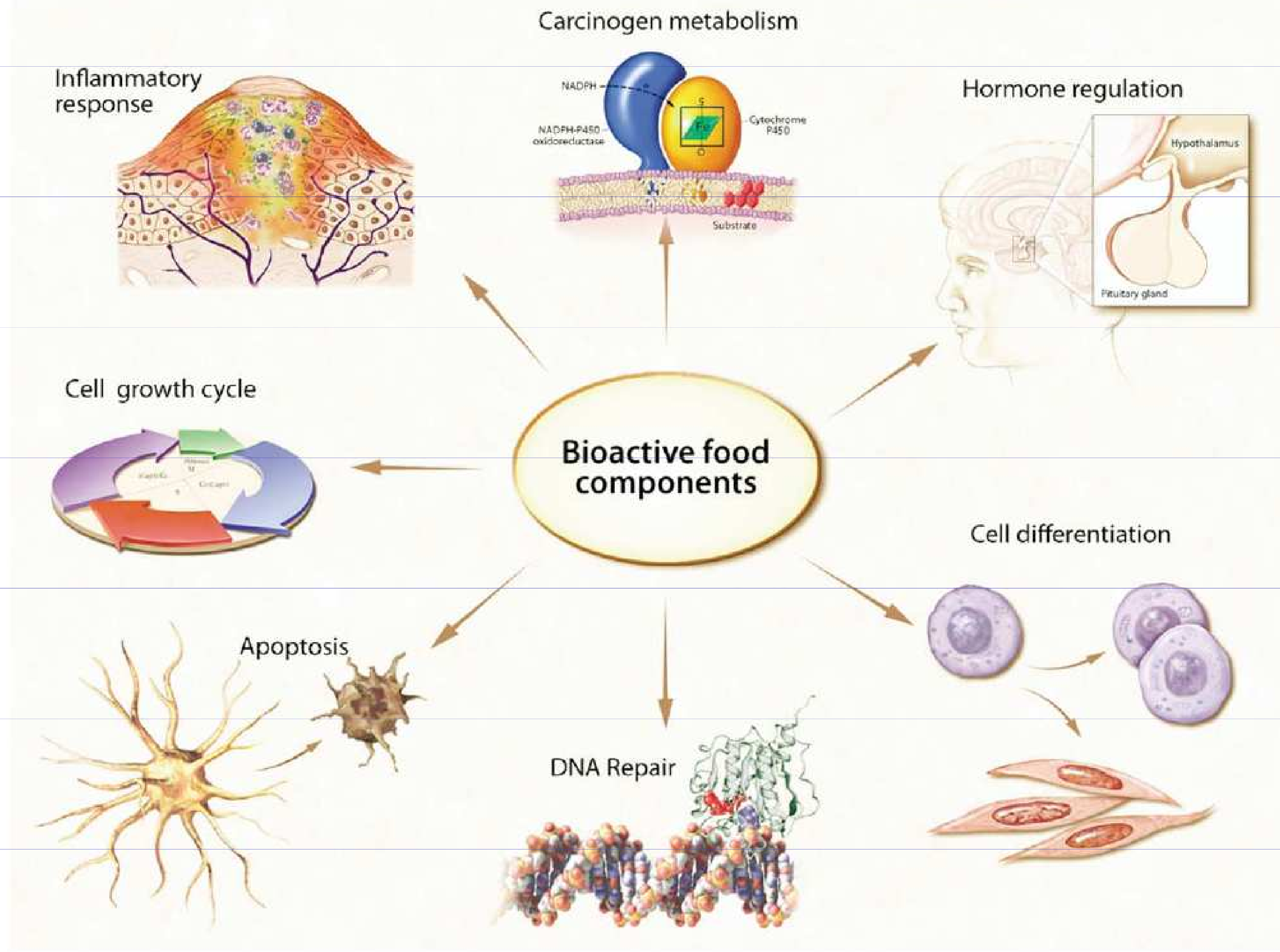
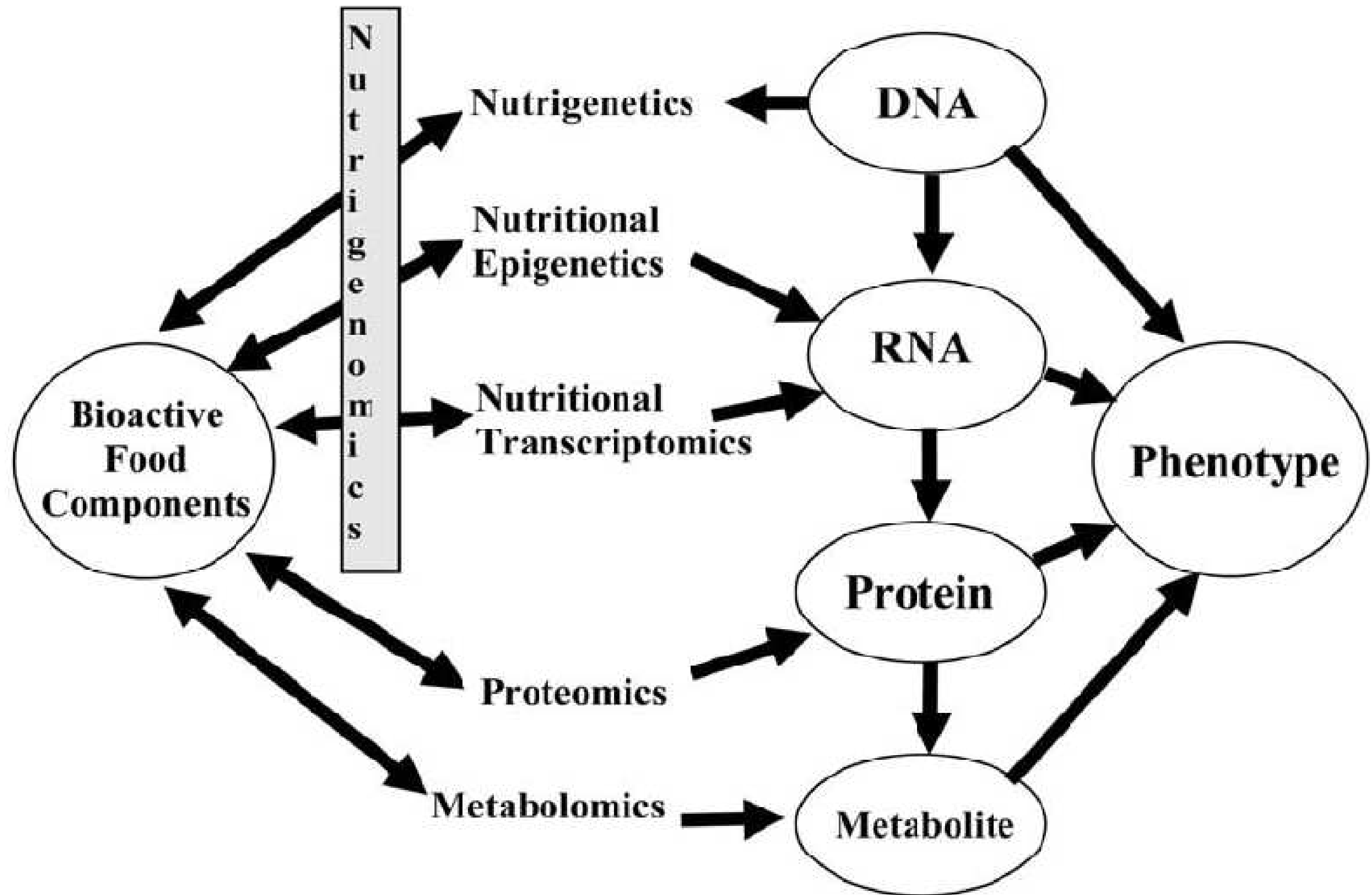


Figure 3. Bioactive food components can influence genetic and epigenetic events associated with a host of disease processes.

Trujillo E., J Amer Diet Assoc., 106, 2006

OMICS ve vztahu k výživě (farmakogenomika, nutrigenomika)

Faktory výživy přispívají různými způsoby k výslednému fenotypu



Using the "omics" of nutrition to identify how dietary factors contribute to establishing a phenotype.

Trujillo E., J Amer Diet Assoc., 106, 2006

VÝŽIVA

hraje roli v mnoha typech onemocnění včetně nádorových a to řadou různých mechanizmů.
Je prokázáno, že vysoký příjem kalorií a tvorba tukových zásob je rizikovým faktorem.

Příjem, absorpce a metabolismus velkého množství potravy vyžaduje oxidativní metabolismus a produkuje více reaktivních kyslíkových radikálů, které poškozují DNA a mají další negativní dopady na metabolismus.

Ukázalo se, že příjem tuků, zejména živočišných zvyšuje riziko kardiovaskulárních a nádorových onemocnění. Epidemiol. studie předpokládají pozitivní korelaci mezi příjmem tuků a nádory prsu, kolonu a prostaty.

Navzdory dlouhé historie studií tuků a nádorů, zůstává řada protikladů. Ukazuje se, že nejen kvantita, ale i kvalita hraje důležitou roli a že se zde uplatňují i tuky rostlinné a rybí olej,
zejména vysoce nenasycené mastné kyseliny (PUFAs) tříd n-3, n-6, olivový olej atd.

LIPIDOVÉ SLOŽKY VÝŽIVY

VÍCE, NEŽLI JEN ZDROJ ENERGIE!!!!

- ◆ strukturální a regulační úloha
- ◆ dopad na fyziologické funkce organizmu
- ► účinky na imunitní systém
- ► regulace proliferace, diferenciace a apoptózy

► úloha v karcinogenezi

(etiologie nádorů tlustého střeva, prostaty, prsu)

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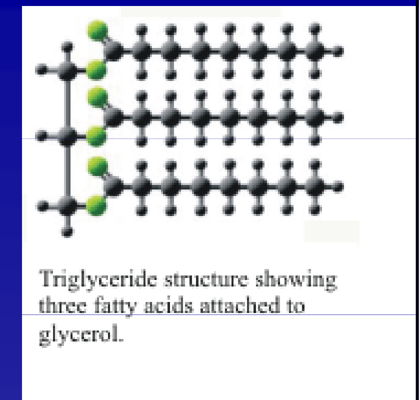

Triglyceride structure showing three fatty acids attached to glycerol.

Změny membránových fosfolipidů přímo ovlivňují **syntézu lipidových mediátorů** typu **eikosanoidů**, PAF a sekundárních přenašečů diacylglycerolu a ceramidu.
Lipidové mediátory ovlivňují produkci a funkci cytokinů.
To má důležitý dopad na řadu imunitních a buněčných funkcí včetně proliferace, diferenciace a apoptózy

Imbalance v lipidovém metabolismu hraje roli u mnoha závažných onemocnění

► Vysoká hladina cholesterolu je spojena s kardiovaskulárními chorobami, které jsou nejčastější příčinou úmrtí v populaci.

► Lipidy produkované buňkami imunitního systému jsou zahrnuty v zánětlivých onemocněních jako je revmatoidní artritida, sepse, astma, zánětlivé onemocnění střeva.

► Lipidy hrají úlohu také v psychických a neurodegenerativních onemocněních (deprese, schizofrenie, Alzheimerova choroba)

► Lipidy ovlivňují počátek a rozvoj nádorových onemocnění

Relativní procento různých mastných kyselin v potravě a změny způsoběné průmyslovým zpracováním potravin

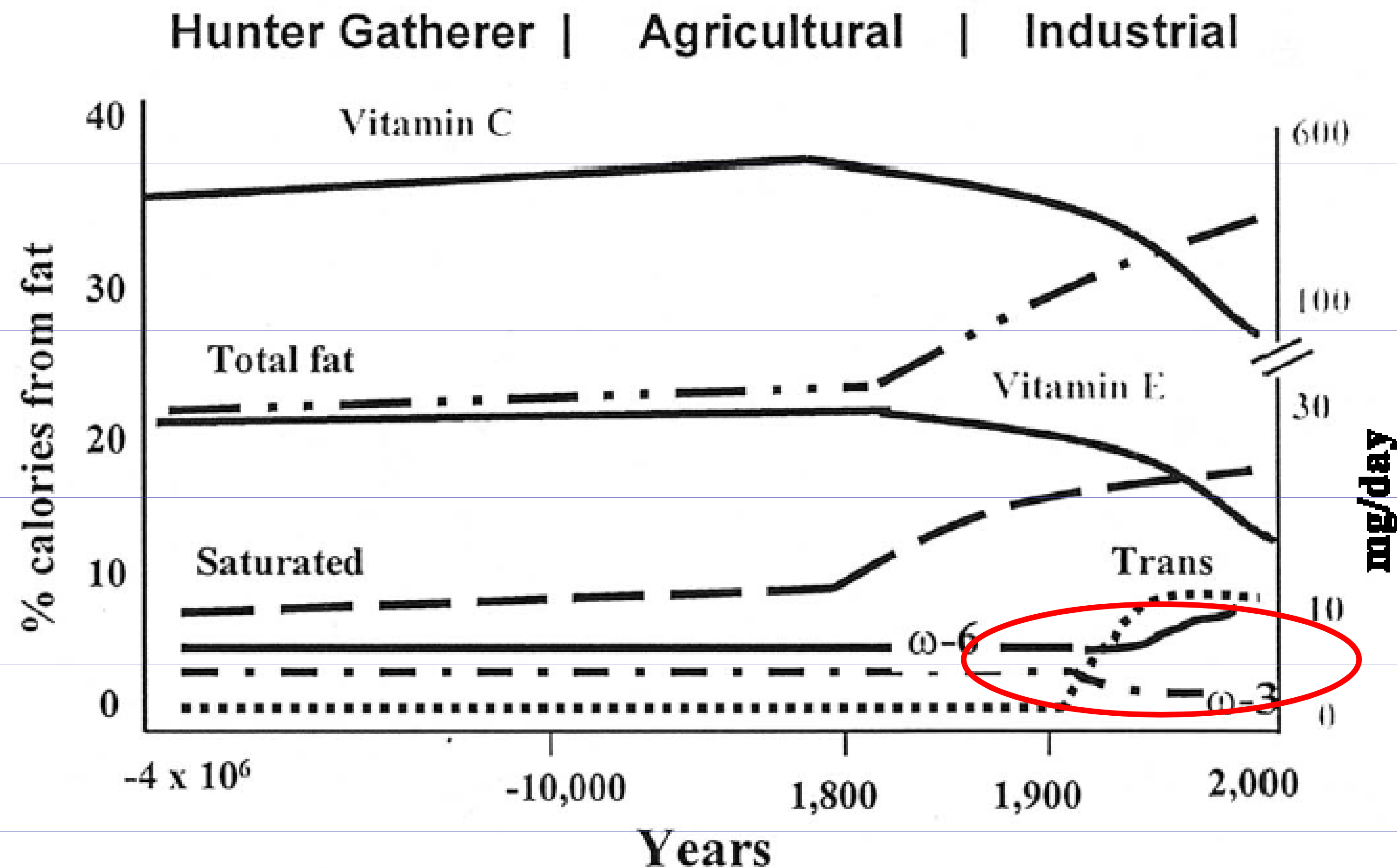
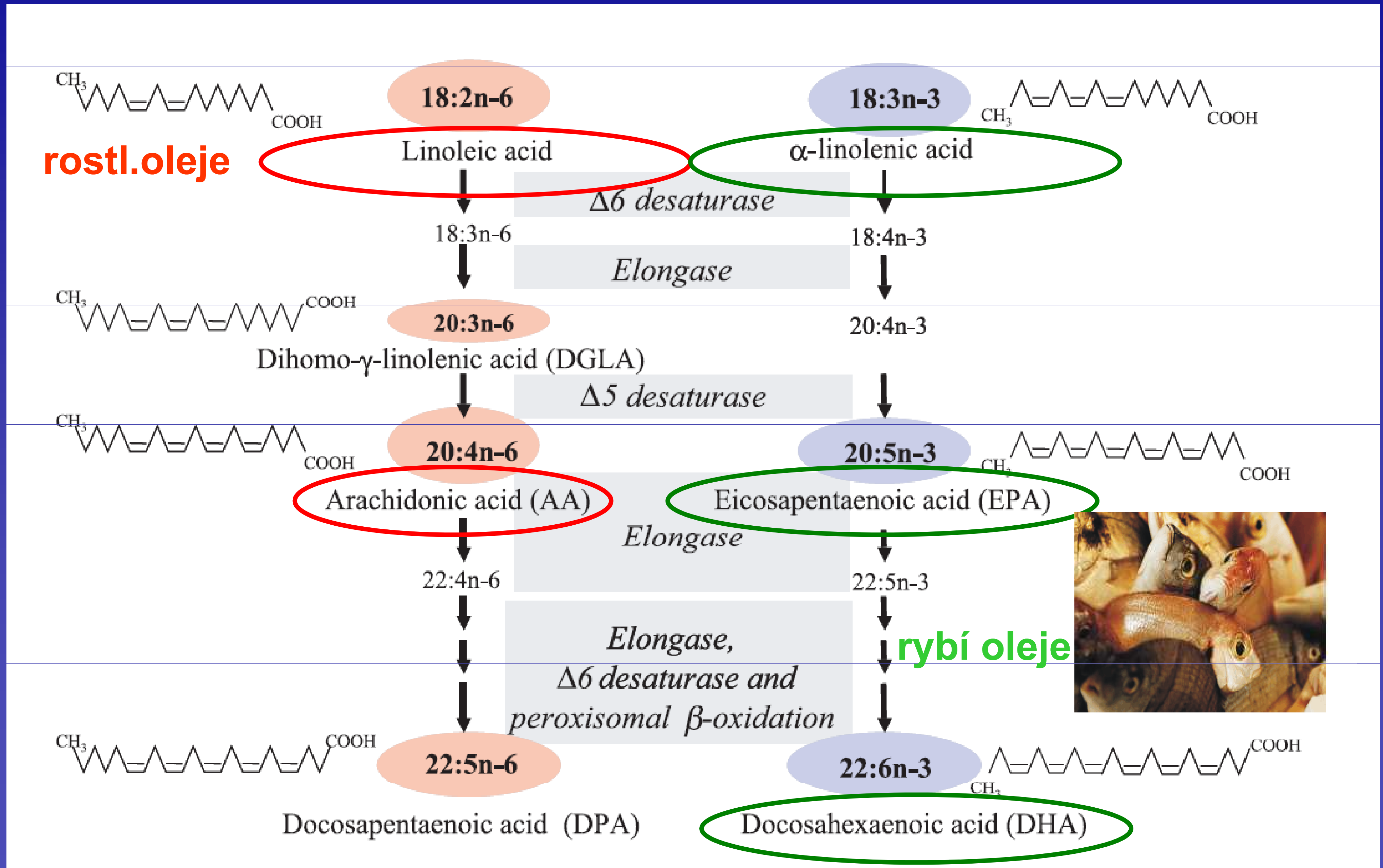


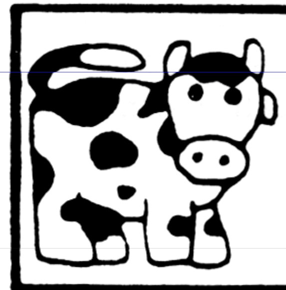
Fig. 1. Hypothetical scheme of fat, fatty acid (ω6, ω3, *trans* and total) intake (as percentage of calories from fat) and intake of vitamins E and C (mg/d). Data were extrapolated from cross-sectional analyses of contemporary hunter-gatherer populations and from longitudinal observations and their putative changes during the preceding 100 years [75].

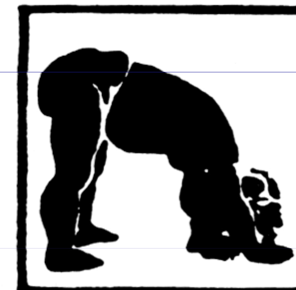
VYSOCE NENASYCENÉ MASTNÉ KYSELINY (VNMK)

(Polyunsaturated fatty acids - PUFAs) - mastné kyseliny s 2 i více dvojnými vazbami. Esenciální prekurzorové kyseliny řady n-6 a n-3



Původ n-3 and n-6 nenasycených mastných kyselin, biosyntéza eikosanoidů z kys. arachidonové a eikosapentaenové

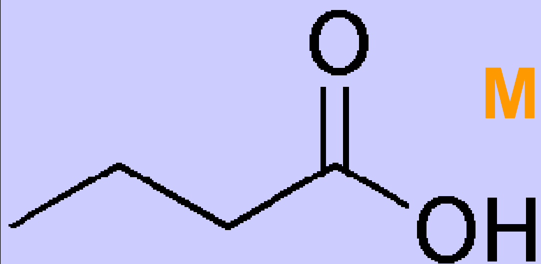




Důležitý je poměr n-3: n-6 VNMK!!!

Kys. linolová (18:2, ω-6)
kyselina arachidonová (AA, 20:4), rostlinné oleje
zdroj eikosanoidů (prostaglandiny, leukotrieny) význam u různých nádorů.
V experimentálních systémech často podpůrný účinek pro vznik a rozvoj nádorů

Kys. alfa-linolenová (18:3, ω-3)
kys. eikosapentaenová (20:5) a dokosahexaenová (22:6)
z rybích a některých rostl. olejů (pupalka, len, rakytník)
V experimentálních systémech často inhibiční účinek pro vznik a rozvoj nádorů



Mastné kyseliny s krátkým řetězcem– BUTYRÁT

► produkován anaerobní mikrobiální fermentací vlákniny ve střevě

► zdroj energie pro normální kolonocyty

► významný pro udržení homeostázy ve střevní tkáni regulací exprese genů spojených s regulací proliferace, diferenciace a apoptózy (microarray analýza – změny exprese 19 400 genů), exportní protein MCT1

► butyrát sodný (NaBt) snižuje proliferaci a indukuje diferenciaci a apoptózu neoplastických kolonocytů *in vitro* a *in vivo*

Prevence NÁDORŮ TLUSTÉHO STŘEVA

► **Výzkum interakce mastných kyselin s endogenními regulátory růstu, diferenciace a apoptózy** (cytokiny, růstové faktory, induktory apoptózy - zejména vliv na cytokinetiku, odhalování mechanizmů) - terapeutické aplikace, lipidové výživy

► **Výzkum interakce VNMK s environmentálními polutanty** (rozpustnost v tucích, aktivace metabolismu lipidů a jejich úloha v působení polutantů - cytokinetika, transdukce signálů, mezibuněčná komunikace)

► **Výzkum interakce VNMK s vybranými farmaky** (mechanismy působení NSAID-nesteroidních antiflogistik, mechanismy a modulace účinků cytostatik - terapeutické aplikace)

Složky lipidového metabolismu v buněčných signalizacích
Mediátory a modulátory

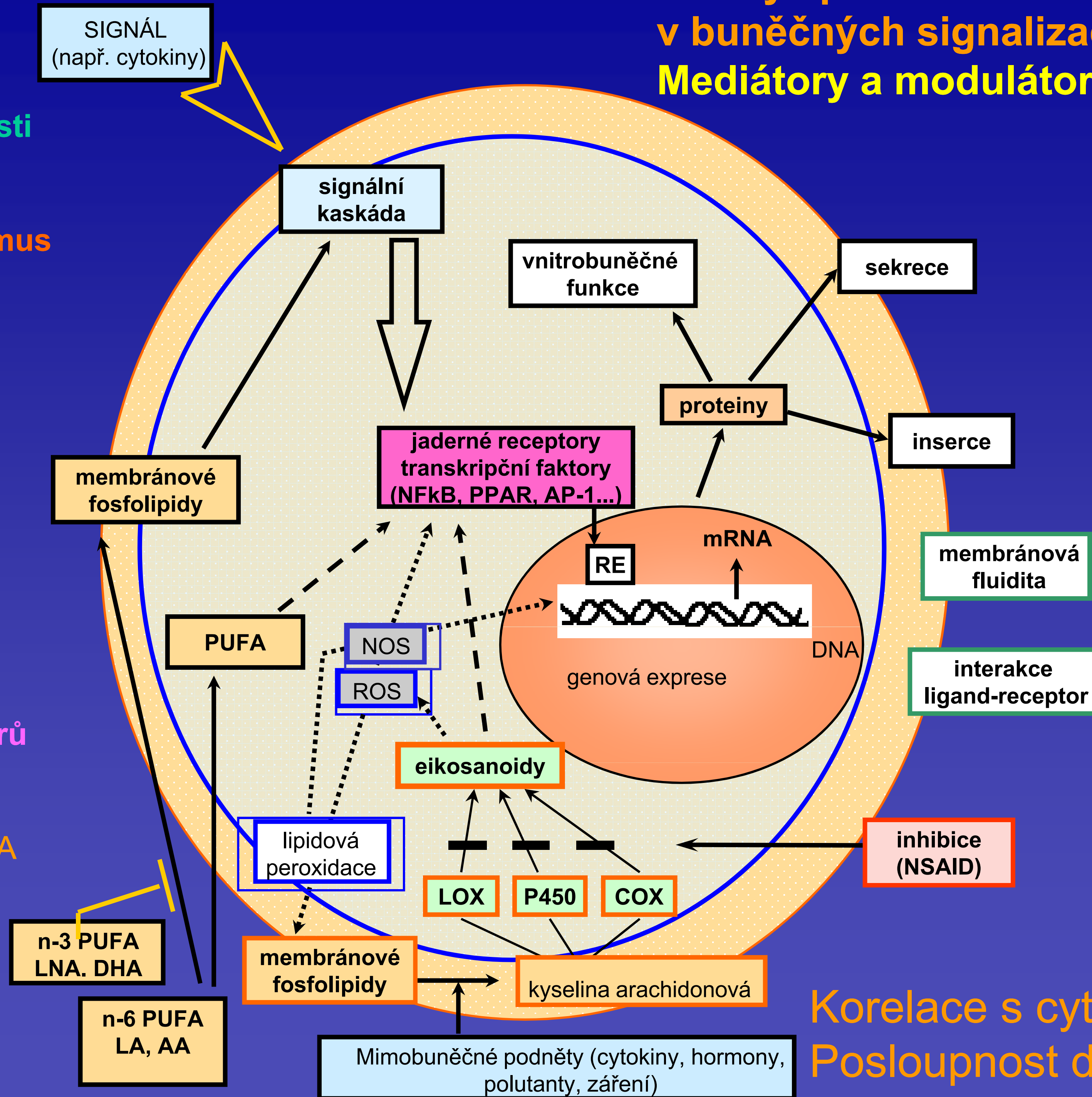
Biofyzikální vlastnosti membrán

Lipidový metabolismus
Aktivace fosfolipáz
Uvolňování a metabolizace AA
eikosanoidy

Oxidativní metabolismus

Transdukce signálů
(kinázy, fosfatázy)
Aktivace membrán. i vnitrobun. receptorů – tr. faktorů

Exprese proteinů
Exprese genů - mRNA



Korelace s cytokinetikou
Posloupnost dějů

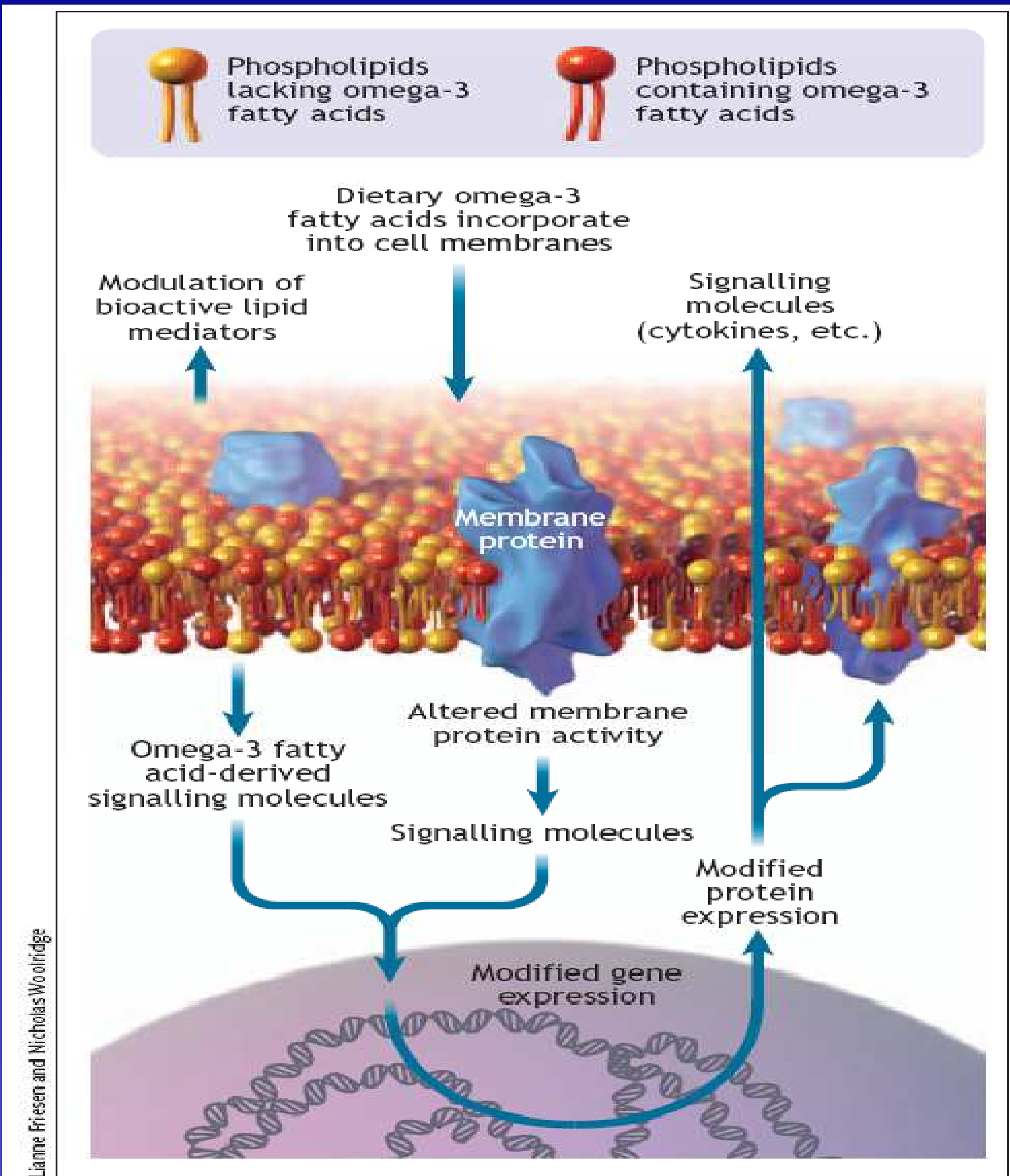


Figure 2: Cell membrane showing omega-3 fatty acids incorporated into the phospholipid bilayer. Omega-3 fatty acids can modify gene and protein expression, modulate membrane protein activity and act as a reservoir for bioactive molecules.

ZMĚNY BIOFYZIKÁLNÍCH VLASTNOSTÍ BUNĚČNÝCH MEMBRÁN

- provázejí procesy diferenciace a apoptózy savčích buněk

- pozorovány rozdíly u
  - nádorových a normálních buněk
  - nádorových buněk senzitivních a rezistentních k cytostatikám

- souvisejí do značné míry s modulacemi ve složení, struktuře, symetrii a metabolismu buněčných lipidů.

Detekce těchto změn
a jejich korelace s dalšími parametry
odrážejícími diferenciaci a apoptózu
přispívá k objasnění

- posloupnosti a regulace jednotlivých kroků těchto dějů
- rozdílů mezi normálními a nádorovými buňkami
- příčin rezistence nádorových buněk k terapii

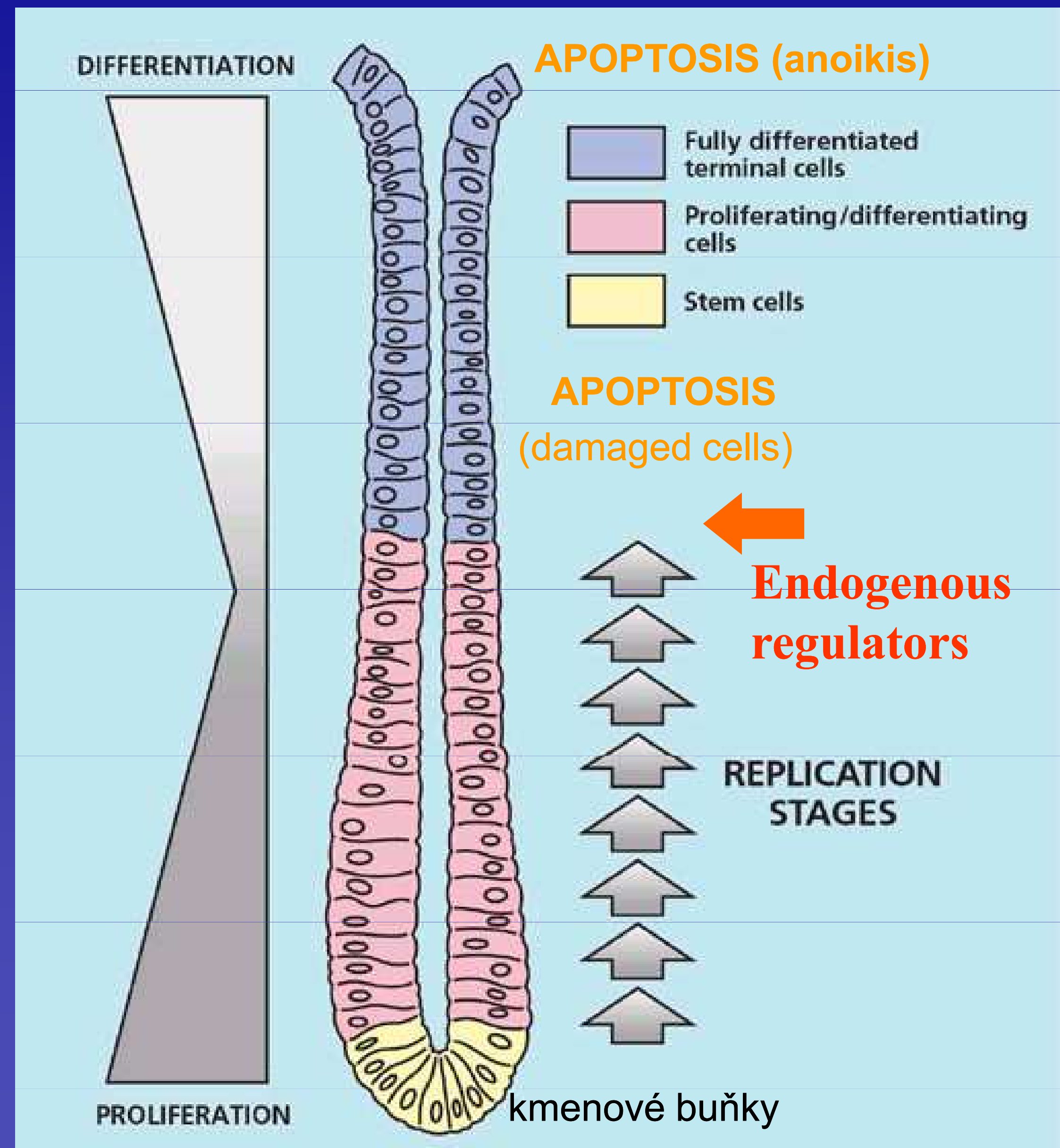
EPITEL TLUSTÉHO STŘEVA (kolonu)

◆ kontinuálně se obnovující buněčné populace

◆ řada zásadních fyziologických funkcí

◆ dynamická rovnováha mezi přírůstkem buněk na bázi krypty (proliferace) a úbytkem (apoptóza-anoikis) na povrchu

◆ regulace endogenními faktory (hormones and cytokines), ale rovněž složkami diety přítomnými v lumen střeva



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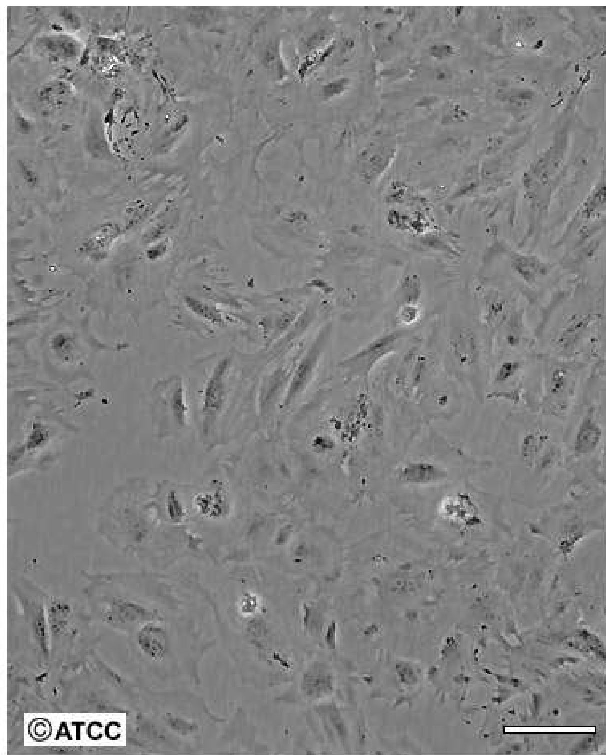
Linie lidských epiteliálních buněk kolonu

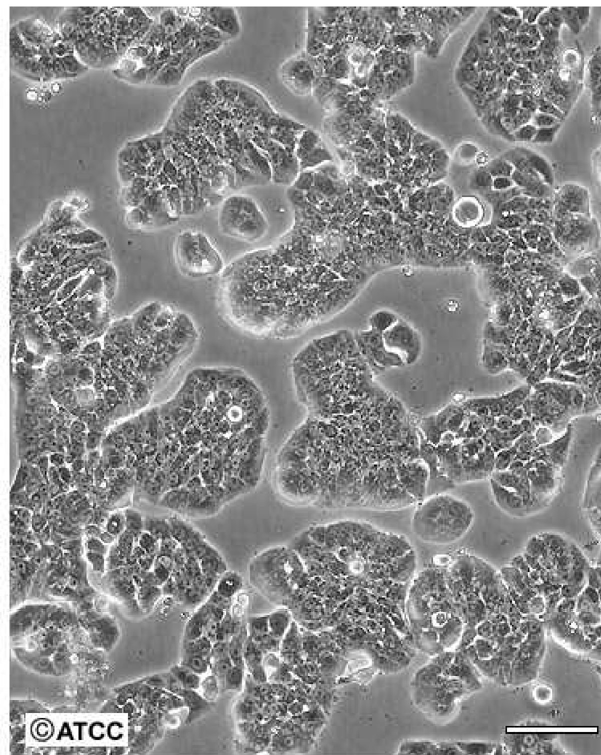
FHC
normální
fetální střevo

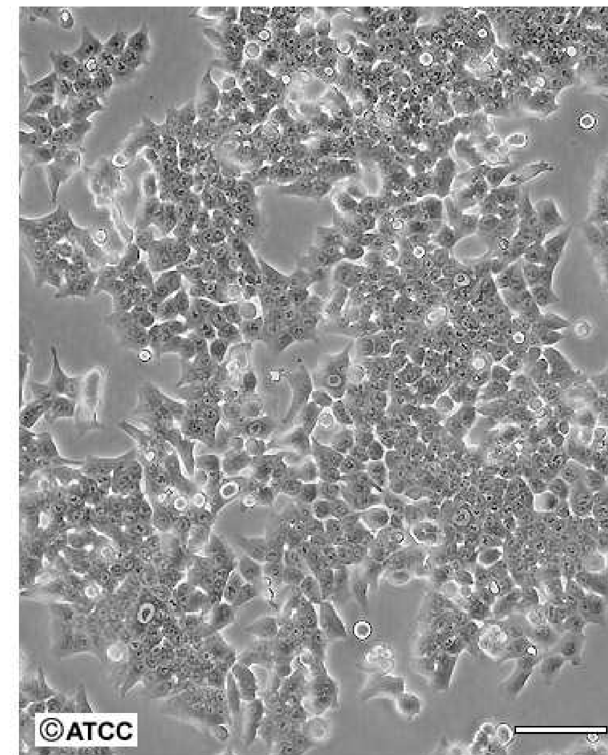
HT-29
diferencující
neinvazívní

HCT-116
nediferencující
invazívní

Adenokarcinom kolonu



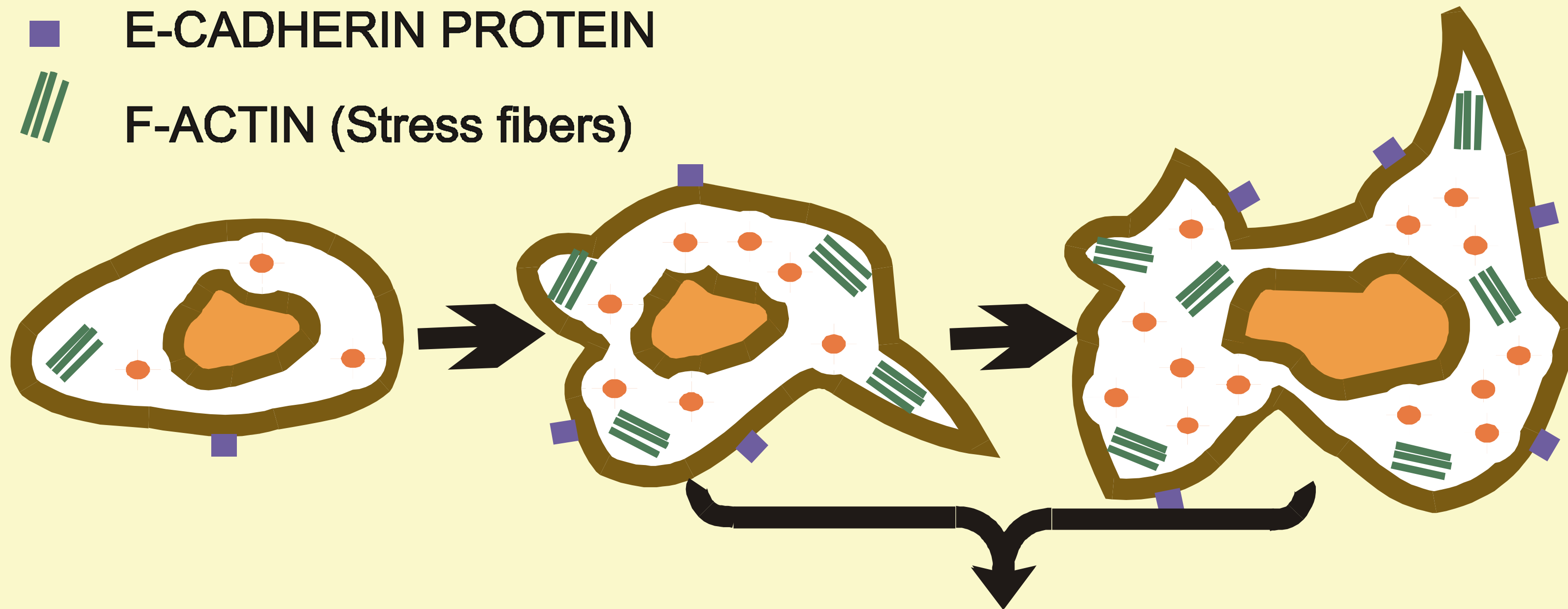


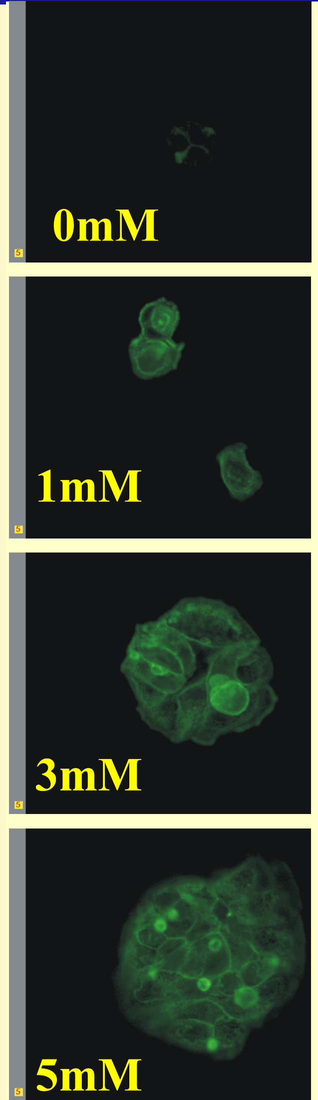


CaCo-2

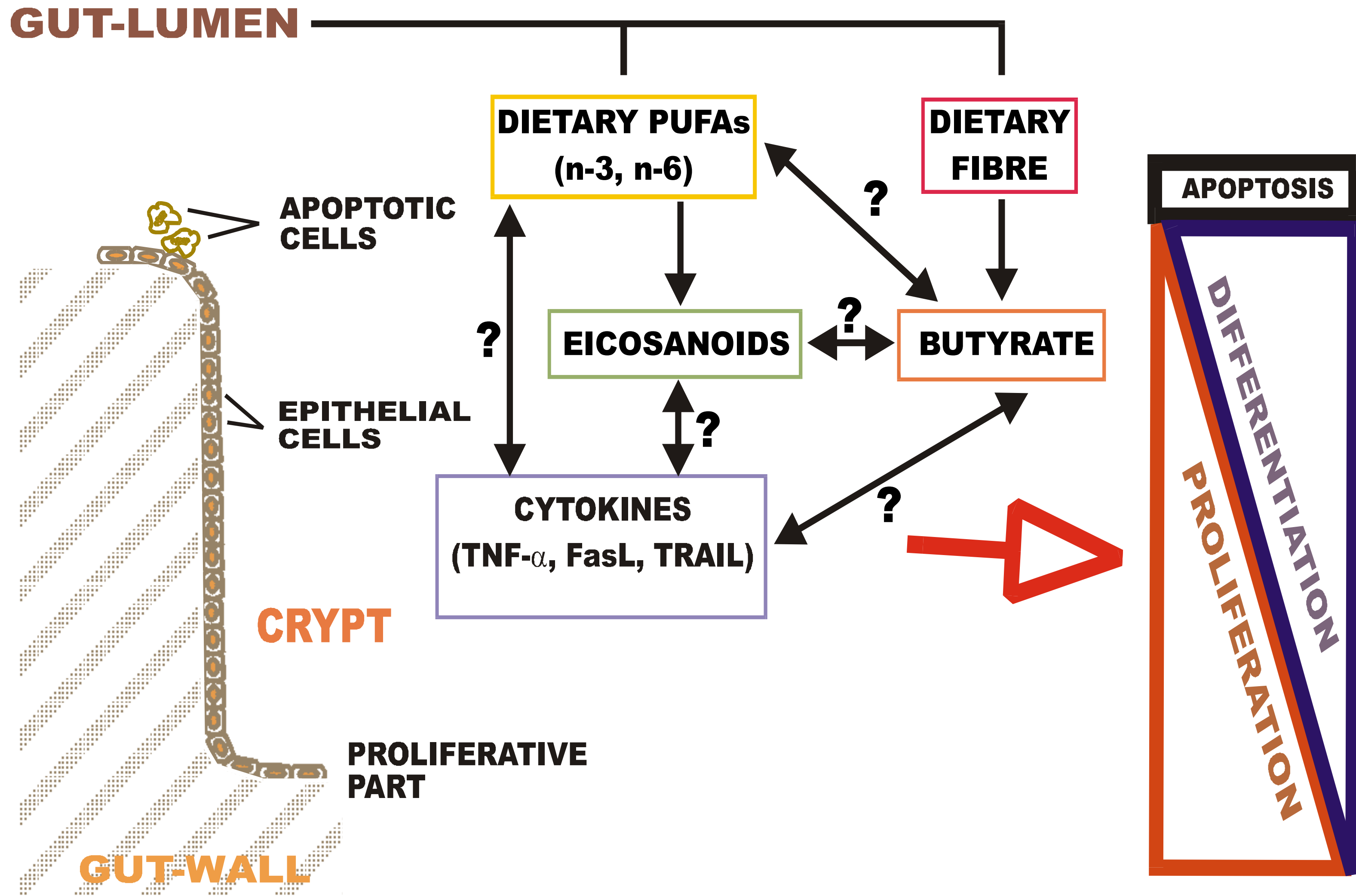
SW 620 – lymf. uzlina

Znaky diferenciace indukované butyrátem





INTERACTIONS OF DIETARY FACTORS AND ENDOGENOUS REGULATORS SUPPOSED TO AFFECT CYTOKINETICS OF COLONIC EPITHELIAL CELLS



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Cytokiny rodiny TNF (tumor necrosis factor)

Ligand	Receptor
TNF-α	**TNFR1, TNFR2**
Fas ligand (FasL)	**Fas (CD95, APO-1)**
TRAIL (TNF-related apoptosis-inducing ligand)	**DR4, DR5, DcR1, DcR2**

Výzkum zaměřen na

► úlohu v regulaci buněčné smrti epiteliálních střevních buněk a

► příčiny rezistence nádorových buněk

Aplikace v nádorové terapii

Zvýšení citlivosti buněk zásahy do signálních drah nebo kombinací s dalšími faktory

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potentiation effects
cycloheximide
actinomycin D
IFN gamma

TNF-α
FasL
anti-Fas antibody
TRAIL
PUFAs ?

TNFR1 R2
FasR (CD95)
DR4 DR5
decoy receptors

Cell membrane

DISC
TRADD
FADD
FADD

TRAF2
RIP
JNK
NFκB

cFLIP
ERK1/2
NFκB ?

TYPE I CELLS
extrinsic pathway

(pro)caspase-8
(initiator)

TYPE II CELLS
intrinsic pathway

Bid
tBid
Bax,Bak
mitochondrion
ROS, ΔΨmt
cytochrome c
Apaf-1
(pro)caspase-9
(initiator)
Bcl2
Bcl_{XL}
Smac/DIABLO
IAPs

(pro)caspase-3,-6,-7
(effectors)
substrate cleavage
DEATH

METODOLOGIE

CYTOKINETIKA

Detekce proliferace- regulace buněčného cyklu a zapojených proteinů,

diferenciace -buněčná morfologie, aktivita specifických enzymů, exprese specifických proteinů

apoptózy -detekce charakteristických změn na úrovni jádra, mitochondrií, membrán, cytoskeletu, exprese regulačních proteinů, štěpení specifických enzymů a substrátů

ZMĚNY LIPIDOVÉHO METABOLISMU A VLASTNOSTÍ BUNĚČNÝCH MEMBRÁN

-změny spektra MK v bun. lipidech, „lipid packing“ v membránách, akumulace triglyceridů, detekce kardiolipinu, membránový potenciál

ZMĚNY OXIDATIVNÍHO METABOLISMU

- produkce reaktivních metabolitů kyslíku (ROS) a dusíku, lipidová peroxidace, účinky antioxidantů

Využití moderních metod průtokové cytometrie, fluorescenční mikroskopie, fluorimetrie, spektroskopie, metod molekulární biologie…

STUDOVANÉ FAKTORY

Mastné kyseliny: kys. arachidonová, *kys. linolová*, *α-linolenová*, kys. dokosahexaenová, *kys. eikosapentaenová, kys. olejová*
butyrát (NaBt)
Lipidové emulze různého složení
Cytokiny: TNFα, anti-Fas, TRAIL
Inhibitory metabolismu AA: indomethacin, COX-2 (refecoxib,….), LOX (baicalein, NDGA)

HLAVNÍ STUDOVANÉ INTERAKCE

- PUFA n-6 a n-3 s rodinou TNF
- PUFA a butyrát
- rodina TNF a butyrát
- rodina TNF a inhibitory metabolismu AA v podmínkách diferenciace NaBt

STUDOVANÉ PARAMETRY

- Cytokinetika (proliferace a bun. cyklu, diferenciace, apoptóza – anoikis)
- Proteiny spojené s regulací těchto procesů
- Proteiny signální transdukce
- Adheze a interakce buňka-buňka a buňka-ECM (testy agregace buněk, migrace buněk, invaze buněk – wound healing assay, exprese specif. kadherinů, integrinů a konexinů, CD44, Matrigel???)
- Oxidativní metabolismus (produkce ROS, NO, lipidová peroxidace, změny GSH), účinky antioxidant
- Lipidový metabolismus a změny biofyzikálních vlastností membrán (akumulace triglyceridů, detekce kardiolipinu, „lipid packing“ v membránách, membránový potenciál, fluidita membrán, obsah fosfolipidů a mastných kyselin v membránách, produkce lipidových metabolitů……)

PERGAMON

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TNF-α modulates the differentiation induced by butyrate in the HT-29 human colon adenocarcinoma cell line

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Abstract

The aim of this study was to determine whether and how tumour necrosis factor alpha (TNF-α) modulates butyrate effects. After the treatment of human colon adenocarcinoma HT-29 cells with sodium butyrate (NaBt), TNF-α or with their combinations we detected cell cycle (flow cytometry), cell proliferation (amidoblack and MTT assays), the amount of dead (floating) and apoptotic cells (flow cytometry and fluorescence microscopy), and the level of differentiation by alkaline phosphatase (ALP) activity (spectrophotometry), relative F-actin content (confocal laser scanning microscopy analysis) and E-cadherin expression (Western blot analysis). Both TNF-α and NaBt decreased cell growth in a dose-dependent manner. After combined treatment of the cells with both agents used, either none or additive effects were observed as compared with NaBt treatment alone. The level of dead and apoptotic cells was dose-dependently increased after this combined treatment. In contrast, TNF-α suppressed ALP activity and F-actin accumulation induced by NaBt. The results suggest that TNF-α does not influence significantly the antiproliferative effects of NaBt but, contrary to its potentiation of apoptosis, it markedly reduces NaBt-induced differentiation of HT-29 colon adenocarcinoma cells. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Tumour necrosis factor alpha; Sodium butyrate; Colon cancer; Differentiation markers; Inflammatory bowel diseases

1. Introduction

Tumour necrosis factor-alpha (TNF-α) has a crucial role in immune and inflammatory processes, as well as in the pathogenesis of many human and animal diseases [1,2]. It is synthesised by macrophages and other cells in response to bacterial toxins, inflammatory products and other invasive stimuli [3]. Prolonged production of TNF-α is associated with cancer and chronic infections. It has been suggested that gut with active injury (e.g. in Crohn's disease or ulcerative colitis) contains an increased number of TNF-α-secreting cells [4]. In extracts of colorectal tumour tissues resected from human patients Numata and associates [5] have detected endogenous TNF-α at levels higher than those in the corresponding normal colorectal tissues. Thus, TNF-α may play a significant role in the modulation of differentiation and proliferation of colonocytes during cancer progression. In addition, TNF-α is a drug under investigation for the treatment of cancer [6].

An important factor which maintains the balance between proliferation, differentiation and apoptosis of intestinal epithelial cells in the crypt is butyrate, a 4-carbon fatty acid, which is formed in the gastrointestinal tract of mammals as a result of anaerobic bacterial fermentation of fibre [7]. It has been shown to inhibit the growth and stimulate the differentiation of normal and carcinoma colonic cells, both *in vivo* and *in vitro* [8,9] at concentrations of approximately 5 mM [10].

In this study, we used an *in vitro* model — the HT-29 cell line, which is particularly attractive since it is one of the cell lines of intestinal origin which reversibly displays structural and functional features of mature intestinal epithelial cells [11]. HT-29 cells under normal culture conditions display an undifferentiated phenotype, but they can express an 'enterocyte-like' differentiated phenotype in response to various inducers of

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ORIGINAL ARTICLE

Martina Kovaříková · Jiřina Hofmanová · Karel Souček · Alois Kozubík

The effects of TNF-α and inhibitors of arachidonic acid metabolism on human colon HT-29 cells depend on differentiation status

Received July 2, 2003; accepted in revised form December 23, 2003

Abstract The level of differentiation could influence sensitivity of colonic epithelial cells to various stimuli. In our study, the effects of TNF-α, inhibitors of arachidonic acid (AA) metabolism (baicalein, BA; indomethacin, INDO; niflumic acid, NA; nordihydroguaiaretic acid, NDGA), and/or their combinations on undifferentiated or sodium butyrate (NaBt)-differentiated human colon adenocarcinoma HT-29 cells were compared. NaBt-treated cells became growth arrested (blocked in G_0/G_1 phase of the cell cycle), and showed down-regulated Bcl-x_L and up-regulated Bak proteins and increased expression of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX). These cells were more perceptive to anti-proliferative and apoptotic effects of TNF-α. Both inhibitors of LOX (BA and NDGA) and COX (INDO and NA) in higher concentrations modulated cell cycle changes accompanying NaBt-induced differentiation and induced various level of cell death in undifferentiated and differentiated cells. Most important is our finding that TNF-α action on proliferation and cell death can be potentiated by co-treatment of cells with AA metabolism inhibitors, and that these effects were more significant in undifferentiated cells. TNF-α and INDO co-treatment was associated with accumulation of cells in G_0/G_1 cell cycle phase, increased reactive oxygen species production, and elevated caspase-3 activity. These results indicate the role of differentiation status in the sensitivity of HT-29 cells to the anti-proliferative and pro-apoptotic effects of TNF-α, AA metabolism inhibitors, and their combinations, and imply promising possibility for novel anti-cancer strategies.

Key words colon cancer · tumor necrosis factor · sodium butyrate · arachidonic acid metabolism · cytokinetics · cell differentiation

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Introduction

Healthy colonic epithelium is an excellent example of a tissue that maintains equilibrium between proliferation, differentiation, and apoptosis of epithelial cells in the intestinal crypt. The human colon adenocarcinoma cell line HT-29 represents a suitable *in vitro* model for investigation of these cytokinetic parameters. These cells display an undifferentiated phenotype under normal culture conditions and can express an "enterocyte-like" differentiated phenotype as an answer to various inducers of differentiation (Siavoshian et al., 1997) such as sodium butyrate (NaBt). Butyrate, a four-carbon short-chain fatty acid, is the product of anaerobic bacterial fermentation of dietary fibre within the colon (Wolin, 1981). It appears that butyrate inhibits the growth and stimulates the differentiation of colon cancer cells both *in vivo* and *in vitro* (McIntyre et al., 1991; Hague et al., 1996). Moreover, butyrate is often used as a therapeutic agent (Velazquez et al., 1997).

During the individual stages of cell differentiation many significant changes occur, including changes in sensitivity to different stimuli such as cytotoxic agents, inducers of apoptosis, or inhibitors of proliferation. The results from our laboratory showed that human myeloid leukemia HL-60 cells became resistant to death receptor-mediated apoptosis during their differentiation (Vondráček et al., 2001a).

One of the important inducers of both necrotic and apoptotic forms of cell death (Laster et al., 1988) is the

TNFα zvyšuje apoptózu buněk HT-29, ale potlačuje diferenciaci indukovanou NaBt

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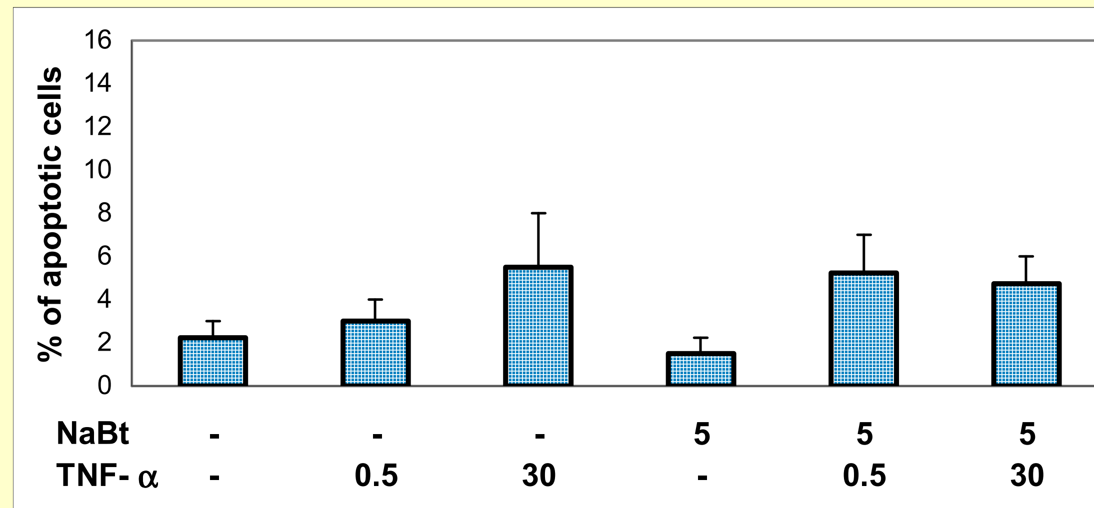
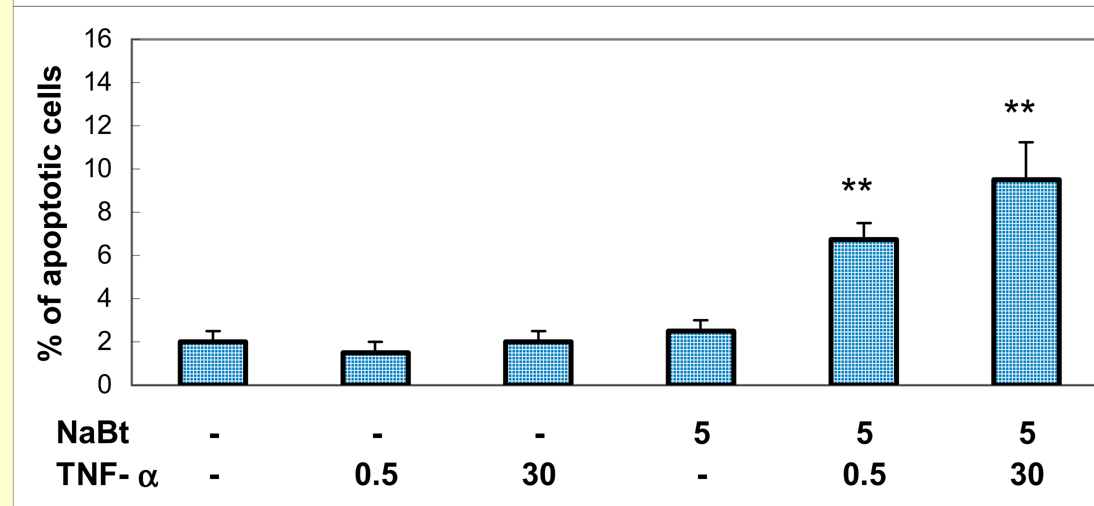
Determination of cell apoptosis
(DAPI staining and fluorescence microscopy)

24 h



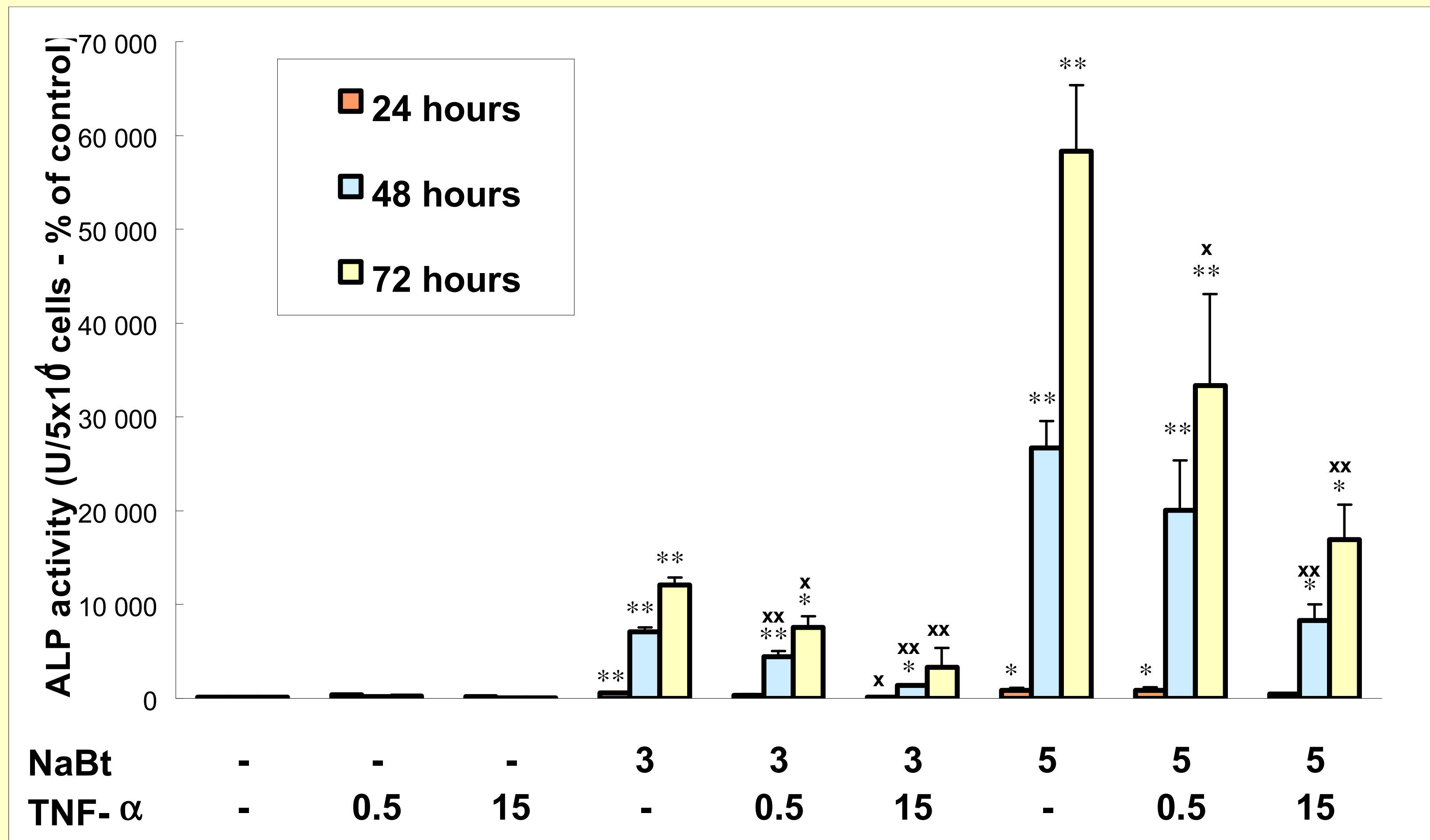
48 h



Laboratory of Cytokinetics

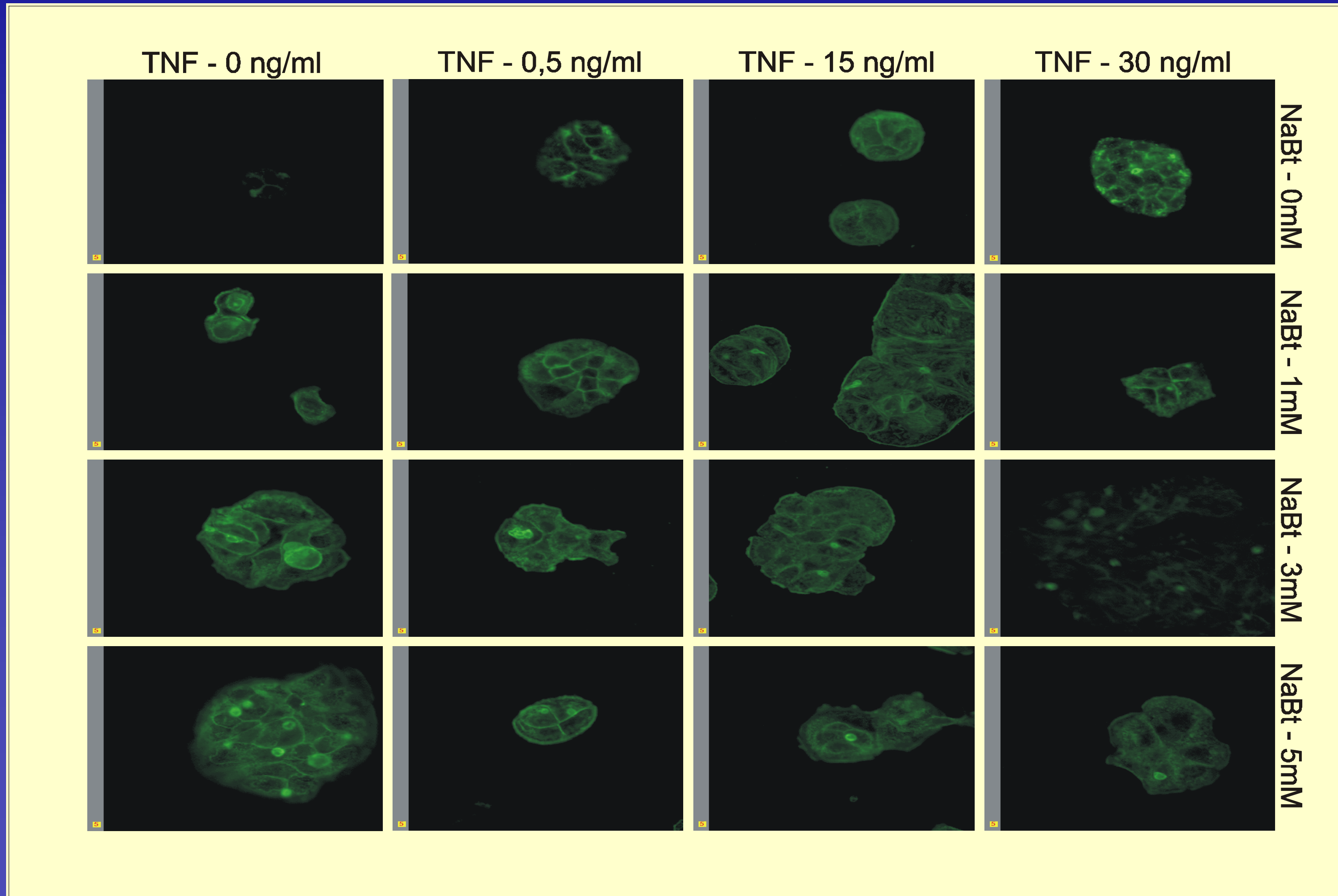
Determination of cell differentiation

(alkaline phoshatase activity)



Laboratory of Cytokinetics

Determination of cell differentiation
(F-actin content - stained with FITC-labelled phalloidin)



Působením NaBt (navození diferenciace) se buňky stávají citlivější k antiproliferačním a apoptickým účinkům TNFα a inhibitorů metabolismu AA

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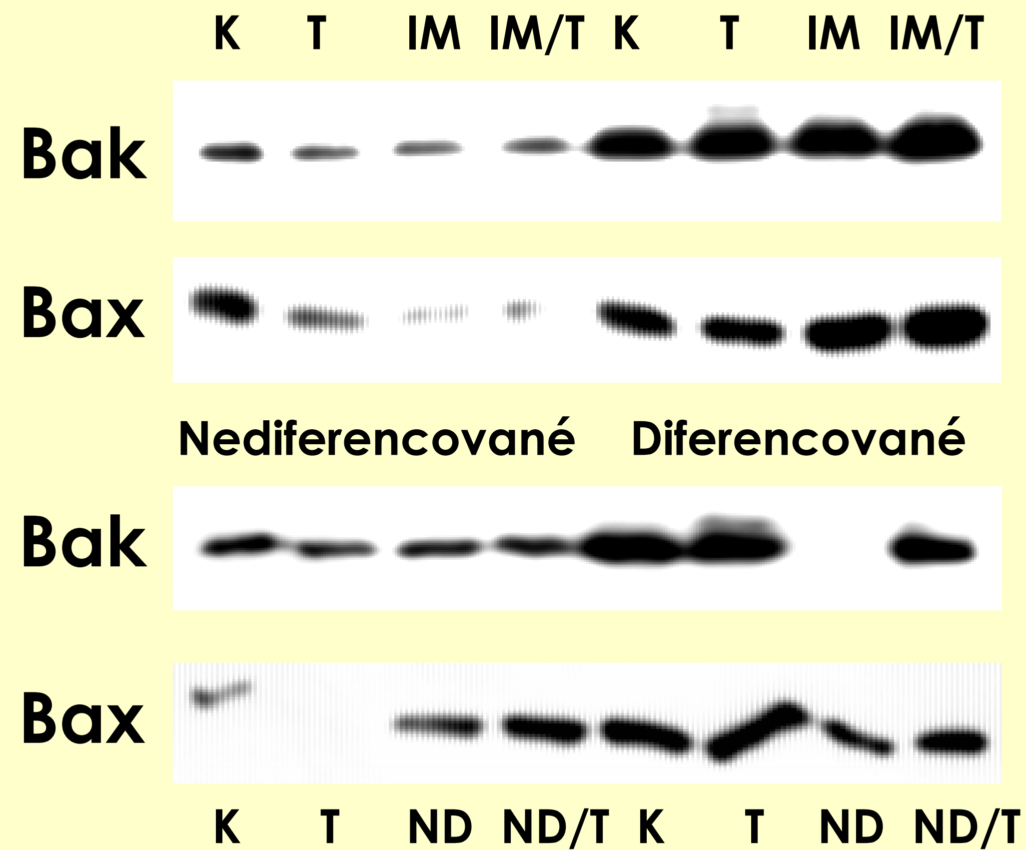
Cytokinetiky
Biofyzikální ústav AVČR, BRNO

Laboratory of Cytokinetics

Exprese proteinů rodiny Bcl



PUFA (kys. arachidonová 20:4, n-6 a kys. dokosahexaenová 22:3, n-3) modulují citlivost buněk HT-29 k působení NaBt a induktorů apoptózy z rodiny TNF

Laboratoř Cytokinetiky

Biofyzikální ústav AVČR, BRNO

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DOI 10.1007/s00394-004-0490-2
ORIGINAL CONTRIBUTION

Jiřina Hofmanová
Alena Vaculová
Antonín Lojek
Alois Kozubík

Interaction of polyunsaturated fatty acids and sodium butyrate during apoptosis in HT-29 human colon adenocarcinoma cells

Summary *Background* Dysregulation of the balance between cell growth and death in the colonic epithelium is associated with cancer promotion. Understanding how cell death in this self-renewing tissue is regulated and how it is influenced by interaction of specific dietary components, especially fat and fibre, could lead to improved treatment and prevention strategies for cancer. *Aim of the study* The effects of two types of polyunsaturated fatty acids (PUFAs) – arachidonic (AA, 20:4, n-6) or docosahexaenoic (DHA, 22:6, n-3) – on the response of human colon adenocarcinoma HT-29 cells to sodium butyrate (NaBt) were investigated. *Methods* The parameters reflecting cell proliferation and cell death were studied together with oxidative response, mitochondrial membrane potential (MMP) and changes of selected regulatory molecules associated with cell cycle (p27Kip1 and p21Cip1/WAF1) and apoptosis (caspase-3, caspase-9, poly (ADP-ribose) polymerase – PARP, Bcl-2, Bax, Bak, Mcl-1). *Results* We demonstrated that pre-treatment with either AA or DHA attenuated cell cycle arrest caused by NaBt which is associated with modulation of p27Kip1, but not p21Cip1/WAF1 protein expression. On the other hand, PUFAs sensitised HT-29 cells to NaBt-induced apoptosis. An increased amount of floating cells and cells in the subG_0/G_1 population was associated with increased reactive oxygen species production, lipid peroxidation, decrease of MMP, activation of caspase-3 and -9, PARP cleavage, and decrease in the expression of anti-apoptotic Mcl-1 protein. The observed effects were modulated by the addition of a protein synthesis inhibitor, cycloheximide, and partially reversed by the antioxidant Trolox. *Conclusions* PUFAs may have beneficial effects in the colon enhancing apoptosis induced by NaBt. Alteration of cell membrane lipid composition and potentiation of oxidative processes accompanied by changes in mitochondria followed by stimulation of apoptotic cascade components play a role in these effects.

Key words colon cancer – diet – polyunsaturated fatty acids – butyrate – apoptosis

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Abbreviations

AA	arachidonic acid
CHX	cycloheximide
CL	cardiolipin
DAPI	4,6-diamidino-2-phenyl-indole
DHA	docosahexaenoic acid
DHR-123	dihydrorhodamine
FCM	flow cytometry
FCS	foetal calf serum
MMP	mitochondrial membrane potential
NaBt	sodium butyrate
PARP	poly(ADP-ribose) polymerase
PL	phospholipids
PUFAs	polyunsaturated fatty acids
ROS	reactive oxygen species
TBARs	thiobarbituric acid reactive substances

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Cancer Letters 218 (2005) 33–41
CANCER Letters
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Polyunsaturated fatty acids sensitize human colon adenocarcinoma HT-29 cells to death receptor-mediated apoptosis

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Abstract

The proliferative and apoptotic response to TNF-α and anti-Fas antibody (CH-11) in human colon adenocarcinoma HT-29 cells was modulated by pretreatment with arachidonic (AA, 20:4, $n-6$) or docosahexaenoic (DHA, 22:6, $n-3$) fatty acids, which alone increased reactive oxygen species production and lipid peroxidation, and decreased the S-phase of the cell cycle. The higher amount of floating cells, subG_0/G_1 population and apoptotic cells detected in pre-treated cells was potentiated by cycloheximide. The effects of CH-11 were associated with activation of caspase-8, -9, and -3, cleavage of poly(ADP-ribose)polymerase-PARP, and decreased mitochondrial membrane potential (MMP), but these parameters were not significantly changed after PUFA pretreatment.
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1. Introduction

Supplementation of cell cultures in vitro or feeding animals with $n-3$ or $n-6$ polyunsaturated fatty acids (PUFAs) led to an increase of these PUFAs in cell membrane phospholipids [1,2] and may influence membrane properties [3]. Moreover, PUFAs and their metabolites, eicosanoids, are considered as important mediators and modulators of the intracellular network of signals [4], they change oxidative metabolism [5] and may have a direct effect on gene expression when activating the specific nuclear receptors and transcription factors [6,7]. Certain PUFAs (especially $n-3$ types) were reported to improve immunological response [8], prevent proliferation and initiate apoptosis [9], kill tumor cells in vitro [10], and inhibit tumor growth in experimental animals [11].

The interaction of PUFAs from dietary fat with naturally occurring endogenous factors regulating the cytokinetics is supposed particularly in the colon where epithelial cells are in direct contact with

Abbreviations: AA, arachidonic acid; CHX, cycloheximide; DAPI, 4,6-diamidino-2-phenyl-indole; DHA, docosahexaenoic acid; FCM, flow cytometry; FCS, fetal calf serum; MMP, mitochondrial membrane potential; NaBt, sodium butyrate; PARP, poly(ADP-ribose) polymerase; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; TBARs, thiobarbituric acid reactive substances; TMRE, tetramethylrhodamine ethyl ester perchlorate.

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Cancer Letters 229 (2005) 43–48

CANCER Letters

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TRAIL and docosahexaenoic acid cooperate to induce HT-29 colon cancer cell death

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Abstract

The resistance of some cancer cells to TRAIL-induced apoptosis is a major obstacle in successful clinical application of this cytokine. Combination treatment with agents capable of sensitising the cells to TRAIL effects is beneficial for new cancer treatment strategies. Docosahexaenoic acid (DHA) is under intense investigation for its ability to affect cancer cell growth and apoptosis. We demonstrated a modulation of TRAIL-induced apoptosis of HT-29 human colon cancer cells by DHA on the molecular (pro-caspase-3, -8, Bid, PARP cleavage) and cellular (cell viability and adhesion) level. To conclude, TRAIL and DHA were shown to cooperate in the induction of colon cancer cell apoptosis.

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Keywords: TRAIL; DHA; Cell death; Colon; Cancer

1. Introduction

The tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of TNF family, is particularly interesting for its unique properties to induce cancer cell death while sparing most normal cells. This implies its use as a promising anti-cancer agent [1,2]. Cross-linking of the TRAIL death receptors DR4 (TRAIL-R1, APO-2) and DR5 (TRAIL-R2, TRICK, Killer) results in activation of caspase-8 at the level of the death-inducing signaling complex (DISC) [3,4]. The apical caspase processing is followed by activation of executionery caspases, e.g. caspase-3, cleavage of death substrates like poly(ADP-ribose) polymerase (PARP), and apoptotic cell death [5].

In colon cancer cells, the TRAIL-induced signal transduction pathway remains poorly defined. There is increasing evidence that a number of cancers including some colon cancer cells are resistant to the effects of TRAIL [6,7]. Sensitivity toward TRAIL-induced apoptosis can be modulated at different levels

Abbreviations: DHA, docosahexaenoic acid; FLIP, FLICE inhibitory protein; MMP, mitochondrial membrane potential; PARP, poly(ADP)ribose polymerase; ROS, reactive oxygen species; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand.

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FEBS 28935

FEBS Letters 577 (2004) 309–313

Ethanol acts as a potent agent sensitizing colon cancer cells to the TRAIL-induced apoptosis

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Edited by Vladimir Skulachev

Abstract Identification of mechanisms of modulation of the TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis is important for its potential use in anticancer therapy. Ethanol can induce cell death in vitro and in vivo by different signalling pathways. Its effect in combination with death ligands is unknown. We investigated how ethanol modulates the effects of TRAIL in colon cancer cells. After combined TRAIL and ethanol treatment, a potentiation of caspase-8, -9, -3 activation, a proapoptotic Bid protein cleavage, a decrease of mitochondrial membrane potential, a complete poly(ADP)ribose polymerase cleavage, and disappearance of antiapoptotic Mcl-1 protein were demonstrated. Ethanol acts as a potent agent sensitizing colon cancer cells to TRAIL-induced apoptosis.

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Keywords: TNF-related apoptosis-inducing ligand; Ethanol; Apoptosis; Colon; Cancer

1. Introduction

Ethanol (C_2H_5OH, ethyl alcohol) has been frequently used and generally accepted as a solubilizing agent for a number of different reagents used in laboratory experiments with cell cultures. However, previous reports have demonstrated a role of ethanol in the apoptotic death of many cell types [1–4]. Apoptosis has also been shown to occur in both experimental and clinical alcoholic liver disease, but the signalling pathways remain not fully understood [5]. In addition to the effects of ethanol itself, it is also necessary to consider its interference with other factors, such as endogenous apoptotic regulators. TNF-related apoptosis-inducing ligand (TRAIL), a member of the tumour necrosis factor (TNF) family, is particularly interesting for its unique ability to induce cancer cell death while sparing most normal cells, which implies its use as a potent anti-cancer agent [6]. Cross-linking of TRAIL death receptors DR4 (TRAIL-R1, APO-2) and DR5 (TRAIL-R2, TRICK, Killer) results in activation of caspase-8 at the level of the death-inducing signalling complex (DISC). Activated caspase-8 then initiates the apoptosis executing caspase cascade [7,8].

However, in many cancer cell types, resistance to TRAIL was developed. We investigated the modulation of HT-29 human colon adenocarcinoma cell sensitivity to TRAIL by ethanol. Our results demonstrated a strong potentiation of TRAIL-induced apoptosis in the presence of ethanol and suggested some of the possible mechanisms involved in the interference of these two agents.

2. Materials and methods

2.1. Culture conditions

Human colon adenocarcinoma HT-29 cells (ATCC, Rockville, MD, USA) were cultured in Mc Coy's 5A medium (Sigma, Germany) with gentamycin (50 mg/l; Sigma) and 10% foetal calf serum (FCS; PAN Systems, Germany) at 37 °C in 5% CO_2 and 95% humidity. The attached cells (24 h after seeding) were treated with TRAIL (human Killer TRAIL, 100 ng/ml) and ethanol (4%) alone or in combination for 4 or 24 h in the medium with 5% of FCS. In the experiments using ethanol (0.1–6%) alone, the cells were treated for 48 h.

2.2. Stable transfections

SSFV.neo plasmids (LTR promoter, Neo resistance, *Eco*RI cloning site) [9] with or without (controls) Bcl-2 (kindly provided by Stanley Korsmeyer) were stably introduced into HT-29 cells by transfection using Tfx™ reagent (Promega, Czech Republic) according to the manufacturer's instructions. The clones were then selected with G418 (0.25 mg/ml, Genetica, Czech Republic). Two stable clones and one control clone with an empty vector were used in the experiments and treated as described above. After 4 h, poly(ADP)ribose polymerase (PARP) and pro-caspase-8 cleavage as well as Bcl-2 protein level were examined using immunoblotting.

2.3. Cell viability assay

Cell viability was determined microscopically by eosin (0.15%) dye exclusion assay from a total number of 100 cells.

2.4. Fluorescence microscopy

The cells treated as described above were stained with 4,6-diamidino-2-phenyl-indole (DAPI, Fluka, Buche, Switzerland) solution (1 μg DAPI / ml ethanol) at room temperature in the dark for 30 min. They were then mounted in Mowiol 4-88 (Calbiochem, San Diego, CA, USA) and the percentage of apoptotic cells (with chromatin condensation and fragmentation) was determined using a fluorescence microscope (Olympus IX70, Prague, Czech Republic).

2.5. Detection of mitochondrial membrane potential

The cells were incubated (20 min) in Hanks' balanced salt solution (HBSS) with 100 nM of tetramethylrhodamine ethyl ester perchlorate

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Abbreviations: DR, death receptor; FLIP, FLICE inhibitory protein; MMP, mitochondrial membrane potential; PARP, poly(ADP)ribose polymerase; ROS, reactive oxygen species; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

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Praktické aspekty

■ Zánětlivá a nádorová onemocnění střeva
■ Farmakonutrice, „Disease specific nutrition“

►Podpůrná a kombinovaná terapie

■ Antiproliferační, diferenciační a proapoptotické účinky
■ Protizánětlivé účinky
■ Posílení imunitního systému
■ Posílení účinků chemoterapeutik a snížení vedlejších účinků

►Optimalizace lipidových výživ

■ Vývoj nových typů lipidových emulzí pro perorální použití pro prevenci a terapii vytypovaných onemocnění

Physiol. Res. 54: 409-418, 2005

The Effects of Parenteral Lipid Emulsions on Cancer and Normal Human Colon Epithelial Cells *in vitro*

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Accepted October 19, 2004
On-line available December 9, 2004

Summary
Differences in lipid metabolism of tumor and normal tissues suggest a distinct response to available lipid compounds. In this study, the *in vitro* effects of five types of commercial parenteral lipid emulsions were investigated on human cell lines derived from normal fetal colon (FHC) or colon adenocarcinoma (HT-29). Changes of the cellular lipid fatty acid content, cell oxidative response, and the cell growth and death rates were evaluated after 48 h. No effects of any type of emulsions were detected on cell proliferation and viability. Compared to the controls, supplementation with lipid emulsions resulted in a multiple increase of linoleic and linolenic acids in total cell lipids, but the content of arachidonic, eicosapentaenoic, and docosahexaenoic acids decreased particularly in HT-29 cells. The concentration of emulsions which did not affected HT-29 cells increased the percentage of floating and subG$_0$/G$_1$ FHC cells probably due to their higher reactive oxygen species production and lipid peroxidation. Co-treatment of cells with antioxidant Trolox reduced the observed effects. Our results imply that lipid emulsions can differently affect the response of colon cells of distinct origin.

Key words
Fat emulsion • Reactive oxygen • Lipid peroxidation • Apoptosis • Tumor cells

Introduction

Lipids in artificial nutrition play an important role in metabolic reactions and immunity of critically ill patients (Adolph 1999). Recently, it has become clear that besides direct nutritional effects, lipids play numerous structural and regulatory roles which may have an important impact on physiological functions in the organism. Different types of fatty acids may influence cell membrane fluidity, receptor mobility and functions, signal transduction as well as eicosanoid synthesis and cytokine production and functions (Grammatikos *et al.* 1994a, Calder 2001, Hong *et al.* 2002). Lipid mediators have a potent impact on a wide variety of cellular responses including cell growth, differentiation and apoptosis (Maziere *et al.* 1999, Rudolph *et al.* 2001). Certain polyunsaturated fatty acids (PUFAs) were found to inhibit tumor growth and cancer cachexia (Tisdale and Dhesi 1990, Petrik *et al.* 2000). Recruitment of specific lipids by tumor cells made them more sensitive to

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Response of normal and colon cancer epithelial cells to TNF-family apoptotic inducers

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Abstract. We compared the response of normal (FHC) and cancer (HT-29) human colon epithelial cells to the important apoptotic inducers TNF-α, anti-Fas antibody and TNF-related apoptosis inducing ligand (TRAIL). The two cell lines did not respond to TNF-α (15 ng/ml), expressed a limited sensitivity to anti-Fas antibody (200 ng/ml) and a different response to TRAIL (100 ng/ml). We studied apoptosis with regard to the changes at the receptor level (DR, DcR and FLIP) and at the level of mitochondria (Bid protein cleavage, Apo2.7 protein expression and caspase-9 activation). Two different approaches were used to sensitize the cells to TRAIL-induced apoptosis: inhibition of protein synthesis (cycloheximide, CHX) and inhibition of the pro-survival MEK/ERK pathway (U0126). While the two cell lines were markedly sensitized to all three TNF family members by CHX, a different degree of response (especially for TRAIL) was obtained when inhibition of the MEK/ERK pathway was achieved. TRAIL-induced apoptosis was significantly enhanced by U0126 co-treatment in the HT-29 cells, but not in the FHC cells. The most significant differences between the HT-29 and FHC cells co-treated with TRAIL and U0126 were demonstrated with regard to the involvement of the mitochondrial apoptotic pathway, suggesting its importance in the regulation of cell sensitivity to the TRAIL-induced apoptosis.

Introduction

The suppression of apoptosis of colon epithelial cells may cause cellular transformation and favour progression at every stage of the adenoma-carcinoma sequence (1). Cytokines of the tumour necrosis factor (TNF) family have been identified as important inducers of apoptosis, but their role in regulating epithelial cell turnover is not fully understood (2). Their effect on colon cancer cells and the associated molecular and cellular mechanisms have yet to be elucidated (3). TNF-α, Fas ligand and TNF-related apoptosis inducing ligand (TRAIL) induce apoptosis by binding to their respective death receptors (DRs) possessing intracellular death domains which recruit certain adaptor molecules to form the death-inducing signalling complex (DISC) activating the apoptotic caspase cascade (4). Through caspase-8 activation, subsequent downstream signals are started, either through the direct activation of effector caspases (type I cells, extrinsic pathway) or by transferring a signal to mitochondria (type II cells, intrinsic pathway) mediated by cleavage of the Bid protein (5). Changes in mitochondria are associated with the activity of pro- and anti-apoptotic proteins of the Bcl-2 protein family and start events leading to the activation of caspase-9, effector caspases, death-substrate cleavage and finally cell death (6).

In spite of the fact that TNF-α, anti-Fas and TRAIL can generate potent antitumour activity *in vivo* and *in vitro* (7,8), many cancer cells are resistant to this type of DR-mediated killing. Moreover, the therapeutic use of the TNF-α/TNFR or Fas/FasL system in cancer treatment has been hampered by severe side effects. In contrast to TNF-α and FasL, TRAIL induces apoptosis in a wide variety of transformed cell lines, but seems to have little or no cytotoxic effect on most normal cells *in vitro* and *in vivo*. The induction of apoptosis is mediated by the interaction of TRAIL with the two death receptors DR4 and DR5, and the mechanism seems to be rather different from that of TNF-α or FasL (9). Due to the selective effects of TRAIL on cancer cells and its ability to induce apoptosis irrespective of p53 status, it may be a safer therapeutic alternative to the other two cytokines (10). In spite of an increase in sensitivity to TRAIL-induced apoptosis during adenoma to colon carcinoma transition being detected (11), many types of cancer cells become resistant to TRAIL (12). Some cells seem to be protected from TRAIL-induced apoptosis by their expression of decoy receptors (DcR1 and DcR2), which do not transduce apoptotic signals, as well as at the level of certain molecules involved in intracellular signalling pathways (13).

In the present study, two different approaches were used in order to sensitize the colon cells to the apoptotic effects of TNF-α, Fas antibody (anti-Fas) and TRAIL. The cell response

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*Contributed equally

Key words: apoptosis, TNF-related apoptosis inducing ligand, TNF-α, anti-Fas, colon

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Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells

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Abstract Epithelial cells can be manipulated to undergo apoptosis depending on the balance between pro-survival and apoptotic signals. We showed that TRAIL-induced apoptosis may be differentially regulated by inhibitors of MEK ERK (U0126) or PI3K/Akt (LY294002) pathway in TRAIL-sensitive (HT-29) and TRAIL-resistant (SW620) human epithelial colon cancer cells. U0126 or LY294002 significantly enhanced TRAIL-induced apoptosis in HT–29 cells, but not in SW620 cells. We report a different regulation of the level of an anti-apoptotic Mcl-1 protein under MEK/ERK or PI3K/Akt pathway inhibition and suggest the mechanisms involved. A special attention was paid to the role of the ERK1/2, Akt, and glycogen synthase kinase 3β.
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Keywords: TRAIL; Mcl-1; GSK; ERK; Apoptosis; Colon

1. Introduction

The tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of TNF family, is particularly interesting for its unique properties to induce death of cancer cells (including colon) while sparing most normal cells [1]. This implies its use as a promising anti-cancer agent [2]. TRAIL induces apoptosis via interacting with its cognate 'death' receptors, DR4 and DR5, which in turn results in formation of the death-inducing signalling complex (DISC), and caspase-8 processing [3,4]. Caspase-8 activation can then result in the activation of two distinct pathways: a mitochondrial-independent pathway (in type I cells) leading to direct caspase-3 activation and subsequent cleavage of death substrates, and/or a mitochondrial-dependent pathway (in type II cells) involving activation of the pro-apoptotic arm of the Bcl-2 family, and the mitochondrial release of apoptogenic factors [5]. TRAIL was shown to induce apoptosis in different colon cancer cell lines. Interestingly, during colorectal carcinogenesis, a marked increase in sensitivity to the apoptotic effects of TRAIL, associated with progression from benign to malignant tumour type has been reported [6]. For this study, we have used the human colon adenocarcinoma cell line HT-29, which we previously characterized as type II cells [7], and SW620 cells, which are resistant to TRAIL-induced apoptosis, as models for colon cancer.

Failure to undergo apoptosis in response to TRAIL treatment may result in tumour resistance. Sensitivity towards TRAIL-mediated apoptosis can be modulated at different levels in the TRAIL signalling pathway. Protein kinase-mediated signalling has been described as an effective way of directing DR signals [8]. In contrast to regulation by inhibitory proteins or TRAIL receptors, phosphorylation-based signalling may occur without requirement of newly synthesized proteins. This could be especially effective under conditions when DR responses need to be rapidly modulated.

Besides its potential to selectively kill tumour cells, the physiological role of TRAIL seems to be more complex. The ability of TRAIL to promote survival and proliferation of non-cancer cells has been reported [9]. Furthermore, in certain tumour cells, simultaneous or consecutive TRAIL-induced activation of apoptotic and pro-survival pathways has been demonstrated [10]. The balance between these pathways is therefore very important in determining the cell fate. Therefore, further studies are necessary to examine the interaction between TRAIL-induced apoptotic pathways and pro-survival pathways in order to predict the effectiveness of TRAIL in cancer therapy.

The mitogen-activated protein kinases (MAPKs) are serine–threonine kinases that are activated by phosphorylation in response to a variety of extracellular stimuli [11]. MAPKs play a central role in the transduction of signals for growth and differentiation and also act as important modulators of various apoptosis-inducing signals. The extracellular signal-regulated kinases (ERK1/2), activated by MEK1/2, phosphorylate and modulate the function of many regulatory proteins. The protective effect of ERK1/2 on DR-induced apoptosis has also been reported [8].

PI3K/Akt is a major signalling pathway which mediates proliferative signals in the intestinal epithelial cells *in vitro* and *in vivo* [12]. In addition, this pathway is an important regulator of cell differentiation and apoptosis [13]. Promotion of cell survival by the activation of this pathway occurs by inhibition of pro-apoptotic or the induction of survival signals [14,15]. PI3K-mediated activation of Akt also results in inhibition of

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Abbreviations: CK18, cytokeratin 18; DR, death receptor; ERK, extracellular signal-regulated kinase; FLIP, FLICE inhibitory protein; GSK, glycogen synthase kinase; MMP, mitochondrial membrane potential; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PARP, poly(ADP)ribose polymerase; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumour necrosis factor

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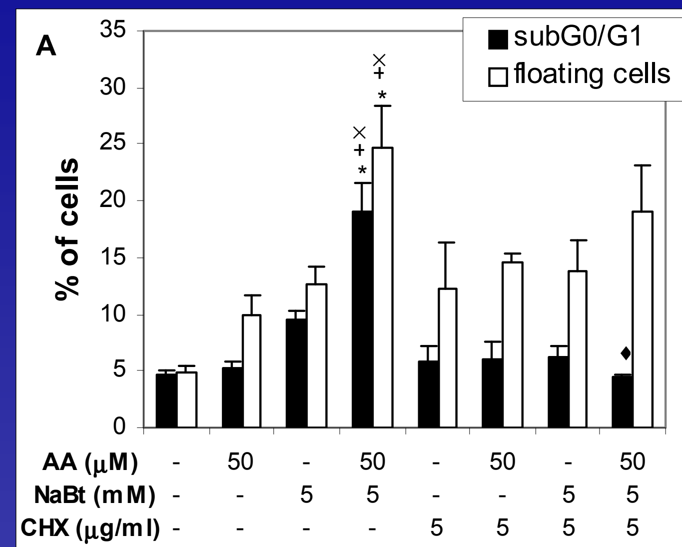
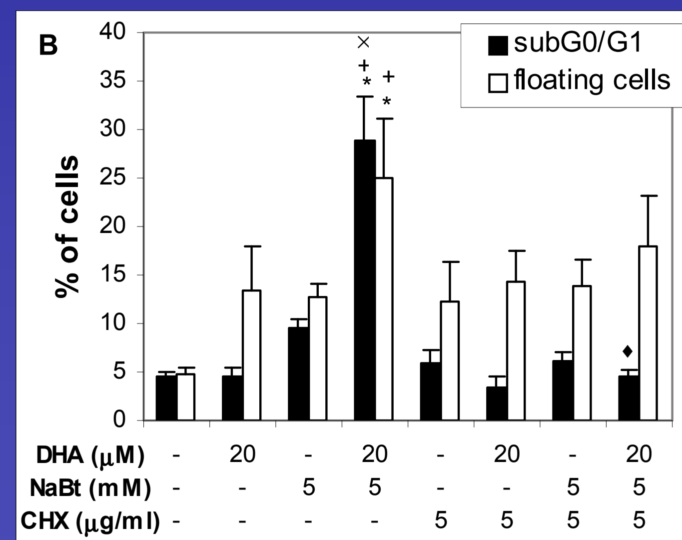
U buněk ovlivněných AA nebo DHA je
po působení NaBt zvýšeno % plovoucích buněk
a subG0/G1 populace.

Spolu s dalšími detekovanými parametry
- ◆ snížení mit. potenciálu (FCM)
- ◆ vyšší produkce ROS (FCM)
- ◆ aktivace kaspázy 3 (Western blot)
- ◆ štěpení PARP (Western blot)

je tak indikována **významně zvýšená apoptóza**
proces **anoikis** – apoptóza indukovaná uvolněním
buněk

Tento efekt je blokován inhibicí syntézy proteinů
cykloheximidem (CHX)





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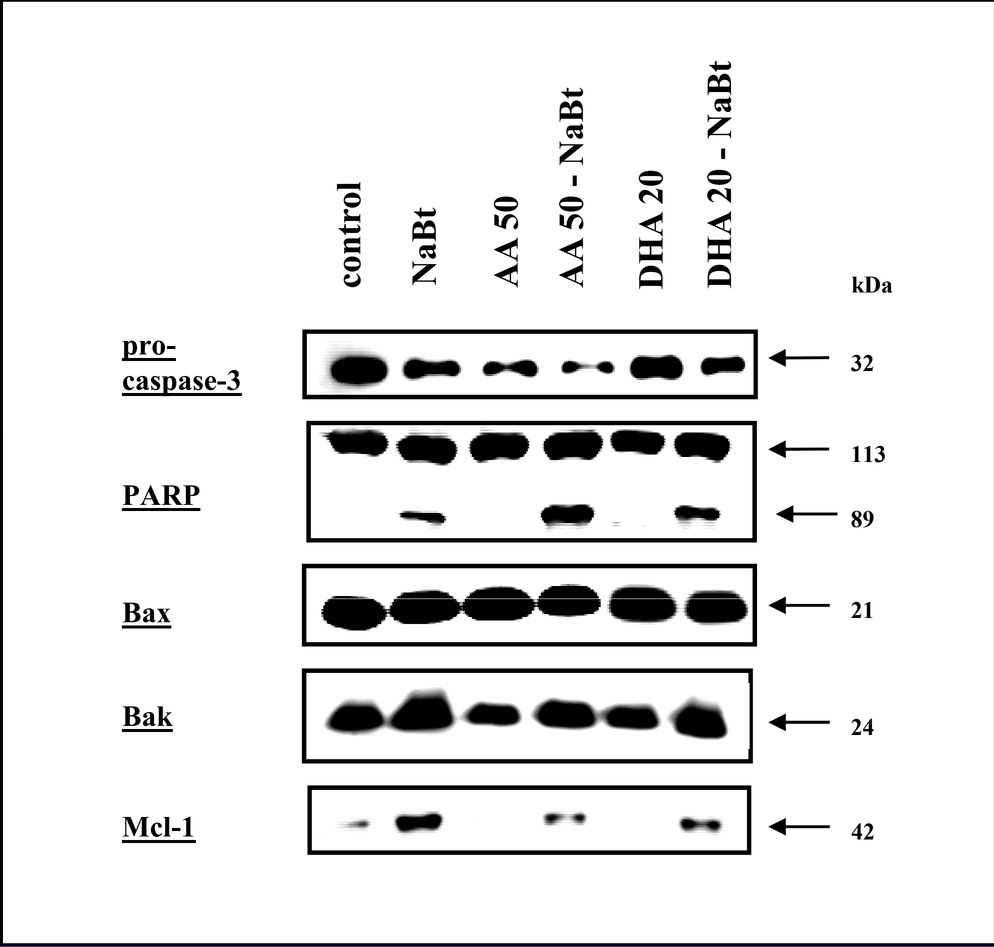
control
NaBt
AA 50
AA 50 - NaBt
DHA 20
DHA 20 - NaBt
kDa
pro-caspase-3
32
PARP
113
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Bax
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Bak
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Mcl-1
42

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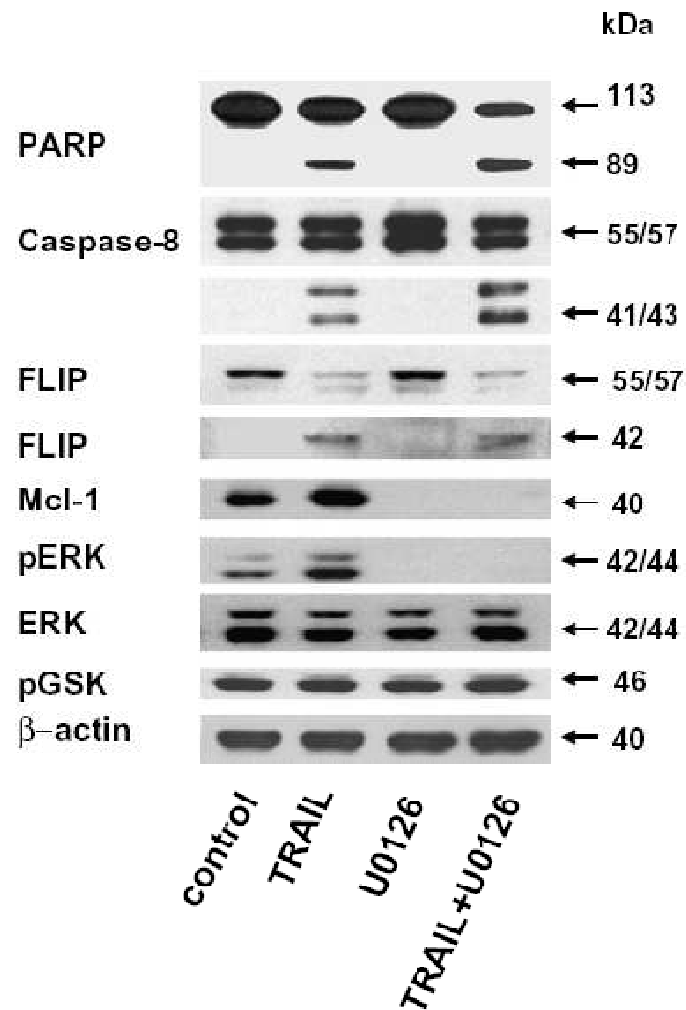


Fig. 3. PARP, pro-caspase-8 and $cFLIP_L$ cleavage, Mcl-1 protein level, phosphorylated and total ERK1/2 levels, and phosphorylated GSK3β in HT-29 cells pre-treated with U0126 (10 μM, 45 min) and then treated with TRAIL (100 ng/ml, 4 h), detected by Western blotting. Results are representative of four independent experiments. An equal loading was verified using β-actin antibody.

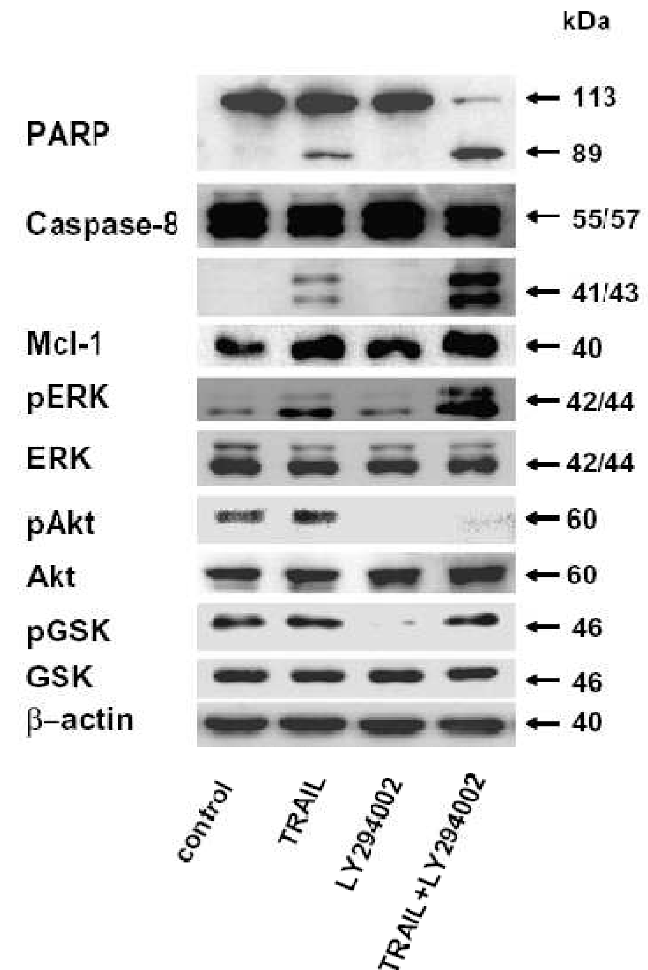
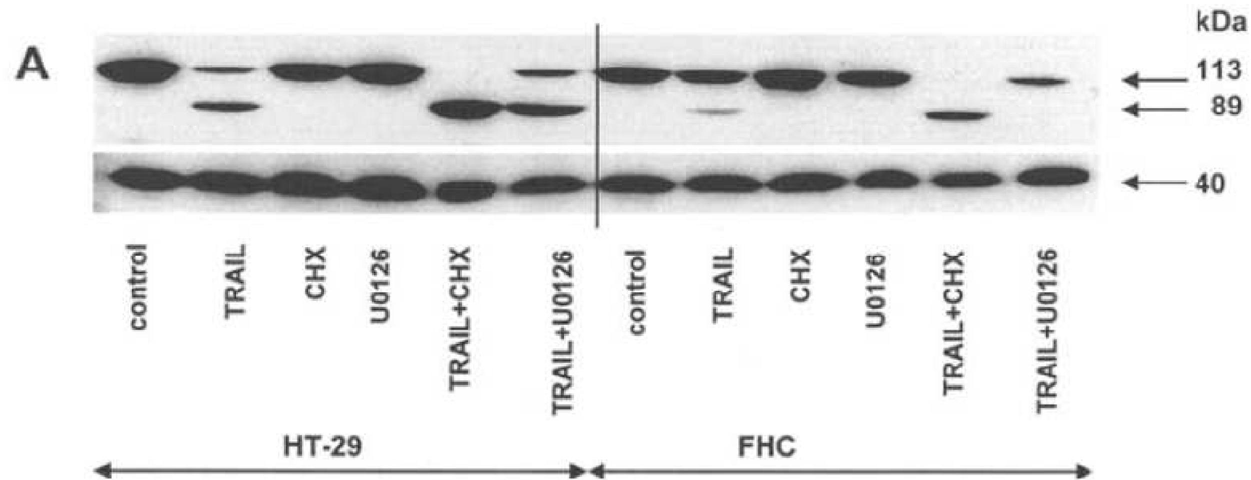
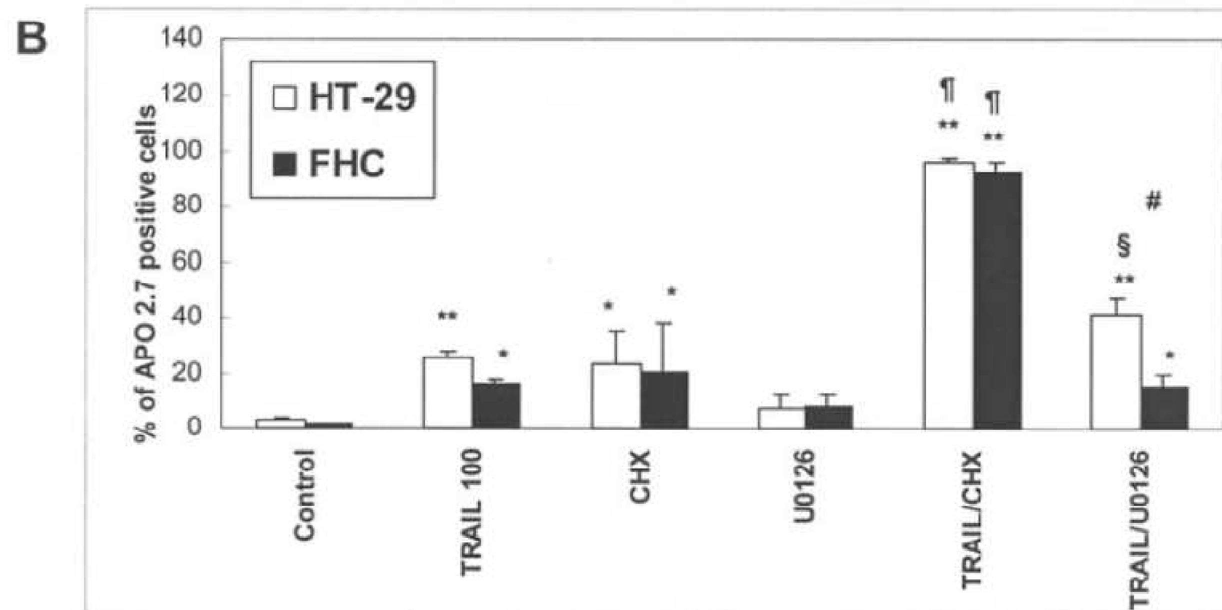


Fig. 4. PARP and pro-caspase-8 cleavage, Mcl-1 protein level, phosphorylated and total ERK1/2, Akt, and GSK3β levels in HT-29 cells pre-treated with LY294002 (50 μM, 60 min) and then treated with TRAIL (100 ng/ml, 4 h), detected by Western blotting. Results are representative of four independent experiments. An equal loading was verified using β-actin antibody.





...vage of PARP and ß-actin quantification in HT-29 and FHC cells treated with TRAIL (100 ng/ml), CHX (5 μg/ml), U0126 (10 μM) or their ...24 h, detected by Western blotting. The results are representative of three independent experiments. (B) Expression of mitochondrial APO2.7 ...itive cells) after 24h treatment of HT-29 and FHC cells with TRAIL (100 ng/ml), CHX (5 μg/ml), U0126 (10 μM) or their combination; ...)1), versus untreated control; §, P<0.05 (¶, P<0.01) versus TRAIL alone; #, between HT-29 and FHC cells.

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Genetics
PODĚBRADKA

Průtoková (flow) cytometrie:

jedna z hlavních používaných metodologií

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