TD4: Endocytosis of LDL and Radioactivity Techniques
Brown&Goldstein, Science (1976), 191, 150

History of study of cholesterol homeostasis

Observation #1 (Barley&Rothblat)

LDL in serum -> cholesterol synthesis decreases
LDL absent -> cholestrol synthesis increases

Method: feed cells radiolabeled acetate- look for incorporation in cholesterol

Observation #2 (Brown&Goldstein)

Relation between LDL and cholesterol synthesis

LDL in serum -> HMGCoA reductase activity decreases
LDL absent -> HMGCoA reductase activity increases

Method: lyse cells, assay for HMGCoA reductase activity

Also:
-No change in sick cells (from hypocholesterolemia patients)
-only LDL gives this effect; other lipoproteins don't
-reversible effect
-very low concentration of LDL required (<10nM)
-delivery of cholesterol by passive diffusion (using EtOH) restores effect in sick cells
Hypothesis-> probably a receptor binds LDL and upregulates HMGCoA reductase, receptor may be damaged or missing in sick cells

Observation #3 (Brown&Goldstein)

Discovery of LDL receptor and endocytosis pathway

1) ^{125}I-labeled LDL sticks to normal cells but not to sick cells
2) ^{125}I-labeled LDL is internalized by cells at 37 deg, not 4 deg, but not by sick cells
3) ^{125}I-labeled LDL is hydrolyzed in lysosomes of normal cells but not sick cells; lysosome inhibitor chloroquine blocks hydrolysis
4) ^{3}H-labeled cholesterol ester in LDL is hydrolyzed in lysosomes

Hypothesis-> probably LDL binds LDL-receptor, gets internalized and targeted to lysosomes, where it is hydrolyzed, releasing cholesterol

MODEL of entire process (summary of many studies)

1. LDL binds LDL receptor (LDL-R)
2. LDL-R clusters, triggering clathrin to form a coated invagination/pit
3. LDL-R/ LDL complex is internalized (endosome)
4. Lower pH of endosome releases LDL
5. endosome fuses with lysosome
6. lysosme enzymes hydrolyze LDL protein (ApoB) and cholesterol esters
7. cholesterol released into cytoplasm-> goes into membranes
8. LDL-R goes back to membrane
9. cholesterol causes: HMGCoA reductase decrease
   ACAT (esterifies cholesterol) increase
   LDL-R decresase

(Net effect: decrease amount of cholesterol in the cell-> negative feedback)

LDL-R makes 1 trip in and out of the cel in 10 min, LDL-R life span is 20 hrs, makes trip >100 times

Focus on radioactivity experiments

Radioisotopes: atoms with the same number of protons but different number of neutrons in nucleus. Chemical properties are determined by # of electrons (which doesn't change), not the overall mass. Hence, isotopes have almost identical properties to non-isotopes -> minimal perturbation -> ideal probes

Ex: ^{12}C: 6 protons + 6 neutrons + 6 electrons (not radioactive)
^{13}C: 6 protons + 7 neutrons + 6 electrons (not radioactive)
^{14}C: 6 protons + 8 neutrons + 6 electrons (unstable= radioactive, emits energy, radiation)

Common isotopes in Biochemistry

Stable Isotopes	Unstable Isotopes	half-life	type of radiation
^{2}H (.015%)	[^{3}H]	12yrs	beta
^{13}C 1.10%)	[^{14}C]	5715yrs	beta
^{15}N (0.37%)	[^{32}P]	14.28days	beta
^{18}O (0.20%)	[^{125}I]	59.4days	gamma

Types of radiation: when radioisotope decays, it can emit:
Alpha particles- (2 protons + 2 neutrons = He nucleus) (not used in biochem)
Beta particles- (electron) fast and light, with medium penetrating power (goes through plastic, stopped by Al or Perspex)
Gamma ray (photon) – high penetrating power- pass through skin and bone, requires lead or concrete to stop

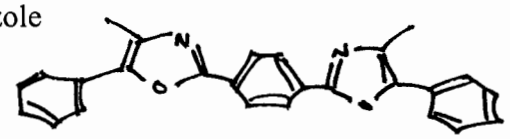
^{125}I is highest energy biological radioisotope, easiest to detect
beta emitter energy differences: $^{32}P > ^{14}C > ^{3}H$

Detection of radioisotopes: scintillation counting, autoradiography, phosphorimaging

Scintillation Counting: convert radiation into visible light with a chemical

3H-> beta particle-> excite solvent (ex. Toluene) -> excite scintillant-> relaxation and photon emission(400-500nm)-> detect using photomultiplier tube (PMT)

a common scintillant is POPOP, an oxazole



-# photons emitted is proportional to the amount of radioisotope (quantitative)
-readout is in cpm (counts per minute)-> convert to moles of radioisotope by first counting a reference standard of known amount
-highly sensitive: 1Ci=$2x10^{12}$cpm, 100 cpm= $5x10^{-11}$ Ci, For ^{3}H compounds, typically, ~50Ci/mmol-> 10^{-12}mmol= 1fmol= 1uL of 1nM ^{3}H compound
Ci (curie)= radioactivity of 1g of radium = 3.7x1010 disintegrations/sec
-worry about quenching
-most sensitive technique, but no spatial information

Place your liquid or solid sample in a glass vial with scintillation cocktail(scintillant and solvent) and put inside LS counter, the machine reads #photons through glass of vial, gives readout in cpm's

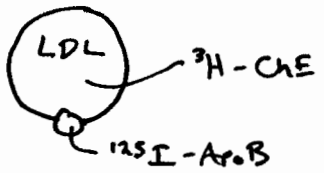
Phosphorimaging (technology owned by Amersham)

Radiolabeled small molecules on TLC plate or radiolabeled macromolecules on gel
Place into a metal cassette that contains a phosphor screen
Wait 10min-days
Read screen with a computer scanner

The phosphor screen contains Eu^{2+} crystals that are phosphorescent, they get excited by radiation and then stay in the excited state for long time. Computer scanner scans screen with a laser that relaxes the Eu^{2+}, causing the release of blue light
-poor for 3H and 14C, beta particles can barely cross the protective coating on the phosphor screen

Autoradiography
Same as phosphorimaging but use a film instead of phosphor screen
Film= $AgCl_2$ crystals suspended in gelatin
$AgCl_2$ + beta particle -> Ag metal deposit
Fix- removes $AgCl_2$ and hardens the gelatin, Ag metal remains

Phosphorimaging is more sensitive and quantitative than autoradiography, larger dynamic range



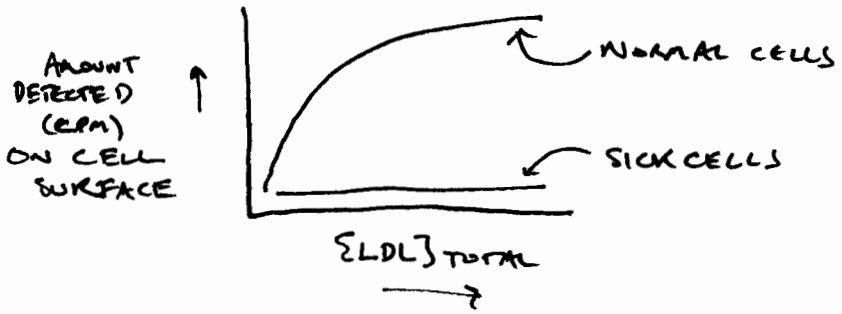
Radiolabeled LDL experiments

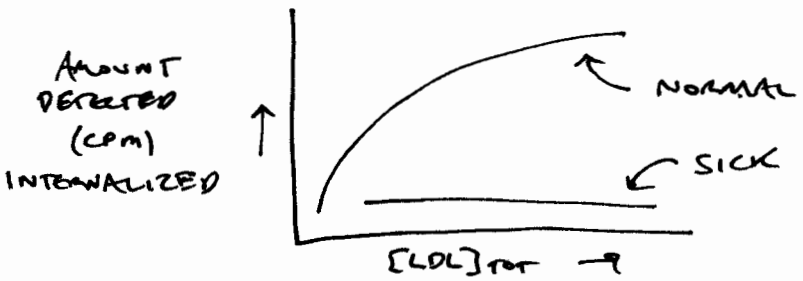
LDL particle is composed of a bag of cholesterol esters (ChE) attached to ApoB protein (radiolabeled with ^{125}I)

Fibroblast (skin) cells are grown in LDL-free media for 2 days
Then add radiolabeled LDL
Wait 2hrs at 37 deg C
There will be LDL bound to receptors on surface, and LDL that has already been internalized
Wash away LDL free in the media
Then wash cells with heparin(sugar), causes the dissociation of surface LDL by competing for binding to LDL
Count washing media: this is the surface bound LDL fraction
Count washed cells: this represents internalized LDL fraction

LDLtot=LDLinside + LDL surface

Observed graph of amt detected (cpm) vs [LDL] total is an increasing saturation curve for normal cells





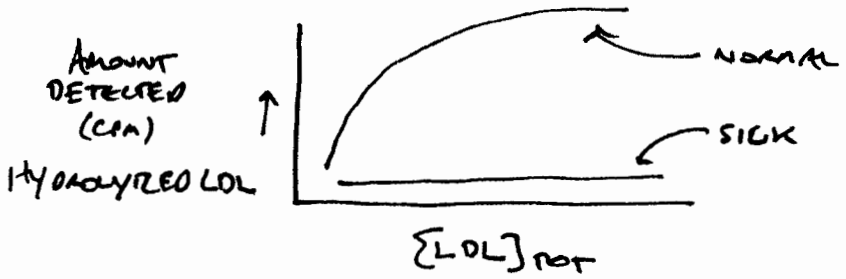
Therefore, LDL sticks to surfaces of normal cells and gets internalized, but not in sick cells

What happens to LDL after internalization?

1. Starve cells (no LDL)
2. add radiolabeled LDL
3. incubate 6hrs at 37 deg
4. lyse cells
5. extract w/ trichloroacetic acid

(intact LDL insoluble in TCA, hydrolysis products –amino acids, proteins, peptides are soluble) -> scintillation count

Amount of TCA soluble radiolabel (hydrolyzed LDL) increases in a saturating curve with [LDL]tot (in normal cells)



After 6 hours, LDL protein gets digested into amino acids in normal cells, but not in sick cells

Same experiment with ^{3}H-ChE containg LDL, but use TLC to detect ^{3}H-cholesterol ester -> ^{3}H colesterol conversion (cholesterol is more hydrophilic than ChE)

Amount of Cholesterol generated from ChE increases in a saturating curve with [LDL]tot