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A Comparison of the Kinetics of Fatty-Acid Metabolism in Smokers and Nonsmokers

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A COMPARISON OF THE KINETICS OF FATTY-ACID METABOLISM IN SMOKERS AND NONSMOKERS

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A COMPARISON OF THE KINETICS OF FATTY-ACID METABOLISM IN SMOKERS AND NONSMOKERS

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I would like to thank Dr. Robert Pawlosky for giving me the opportunity to do this research, Dr. Kate Graham for encouraging me to take the opportunity, and my friends and parents for supporting me through it all.

For many years, the United States government has required labels on all cigarette packages warning against the many ill effects associated with smoking. One of the major factors contributing to the unhealthy aspects of smoking is its profound impact on nutrient levels within the body. Free radicals found in both the gas and tar phases of cigarette smoke may pose a significant threat to the fatty-acid levels in the body. One method of assessing this threat is to examine the fatty-acid synthesis rates of smokers and nonsmokers. An assumption was made that a destruction of fatty-acids will be displayed as an increased rate of synthesis of longer-chain fatty-acids from shorter-chain precursors. A significant difference was found between the fatty-acid metabolic rates of smokers (n=10) and nonsmokers (n=8) with the smokers having an overall increase in rate. Further studies are required to determine if this difference in rate is due to lipid peroxidation brought about by smoking-induced free radical attack.

Introduction:

For many years the surgeon general has warned about the consequences of smoking as they relate to pregnancy, cancer, and heart disease and the recent onslaught of charges against tobacco manufacturers has brought much attention to smoking and its effects on human health (1). Many of the health risks associated with smoking may be attributed to the high concentration of free-radicals found in both the gas and tar phases of cigarette smoke. Free radicals can have devastating effects on lipids, carbohydrates, proteins, and DNA (2). A means of determining the presence of these free-radicals as well as examining their effects is through an analysis of smoking's effects on fatty-acid synthesis and lipid peroxidation.

Lipids are generally characterized as compounds which are insoluble in water but soluble in a nonpolar solvent such as chloroform. Most lipids are derived from fatty-acids-- bipolar compounds with a polar carboxyl head and a non-polar, straight-chain tail (3). Fatty-acids can be saturated (no double bonds in the tail) or unsaturated (one or more double bonds in the tail). Saturated fatty-acids are usually solid at room temperature because they possess the ability to stack on top of each other and, through the attractive forces of London dispersion forces, form weak bonds which therefore increase the melting point. Unsaturated fatty-acids do not readily stack on top of each other due to kinks in the chain at the sites of unsaturation. The double bonds of polyunsaturated fatty-acids are usually separated by a methylene group although some polyunsaturated fatty-acids exist that have conjugated double-bond patterns (3).

The nomenclature used when discussing fatty-acids can be understood by studying Figure 1 which depicts both a saturated and an unsaturated fatty-acid. The saturated fatty-acid is stearic acid or C18:0. This nomenclature means that there are 18 carbons in the fatty-acid and 0 sites of unsaturation. The unsaturated fatty-acid is linolenic acid or C18:3n3. This fatty-acid is composed of

18 carbons and has three sites of unsaturation which begin at the n3 carbon or the third carbon from the non-carboxyl end of the fatty-acid. The other two double bonds are spaced by methylene groups and therefore occur at the n6 and n9 carbons.

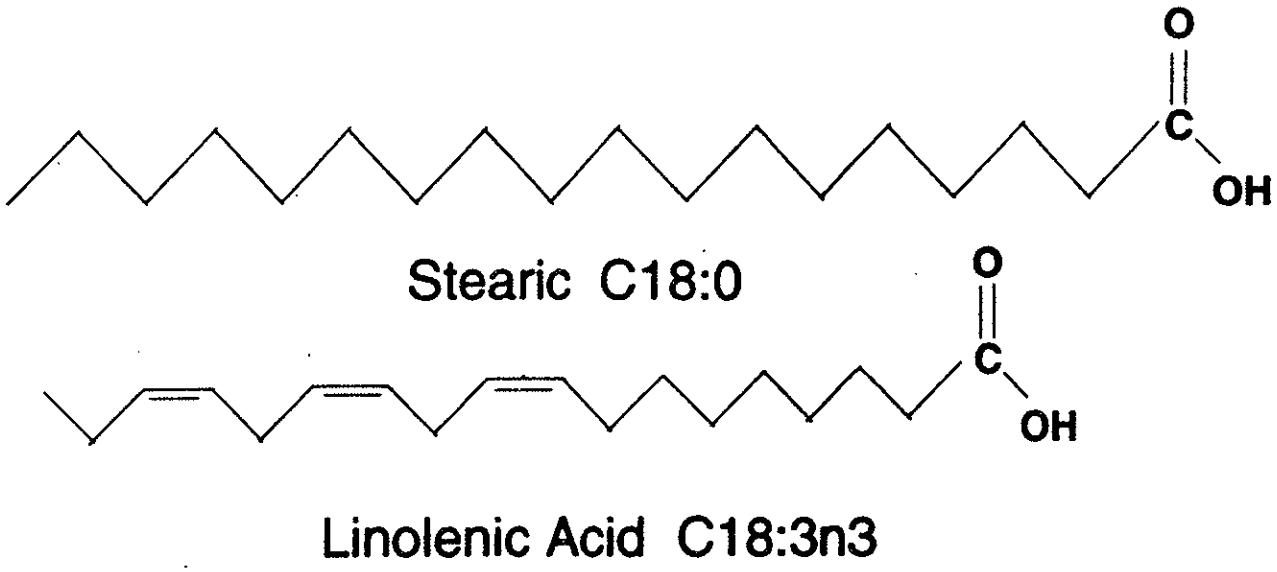


Figure 1. A depiction of two fatty-acids. Stearic acid is a saturated and linolenic acid is unsaturated.

Fatty-acids can originate from two sources; they can be ingested as part of the diet or they can be synthesized from precursors found in the body (4). Two important fatty-acids cannot be synthesized by animals and are therefore considered to be 'essential fatty-acids' because they must be included as part of the diet. These are linoleic acid (C18:2n6) and linolenic acid (C18:3n3). If these essential fatty-acids are completely absent, a person may suffer from

retarded growth, dermatitis, kidney lesions, and early death (4). The conversion of linoleic and linolenic acids to arachidonic and eicosapentaenoic acids involves a series of elongation and desaturation steps (Figure 2).

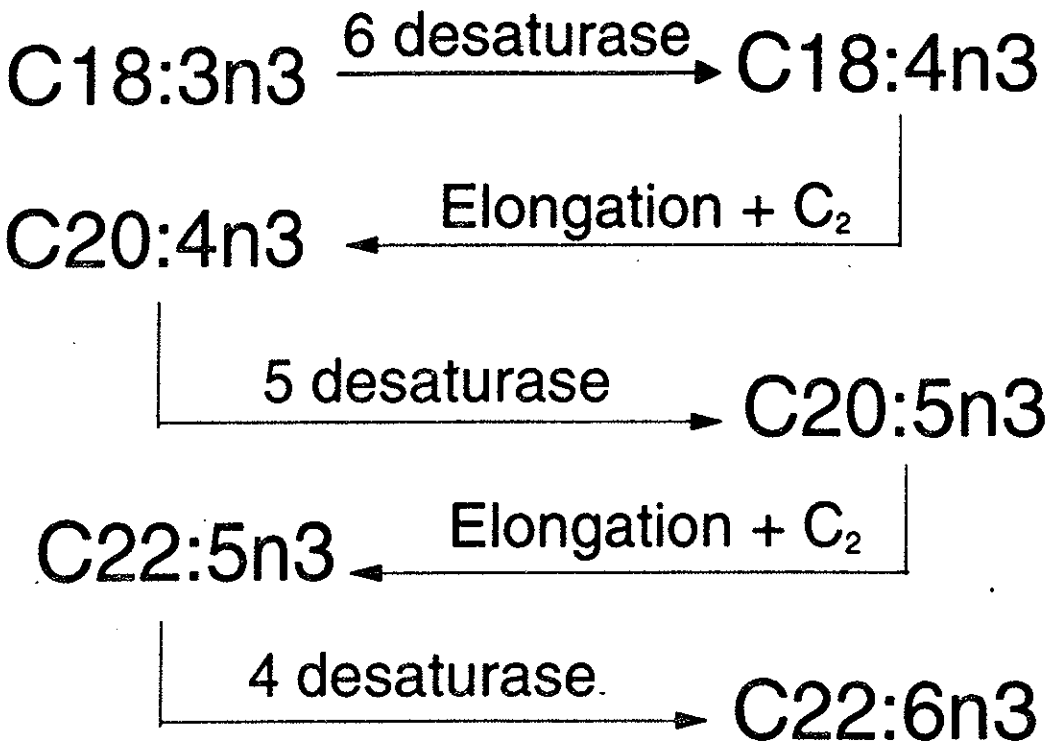


Figure 2. An illustration of the n3 fatty-acid synthetic pathway.

The fatty-acids in the body, including arachidonic and eicosapentaenoic acids, are generally stored in biological membranes until they are needed elsewhere in the body. Phospholipids containing esterified fatty-acids are the main component of cell-membranes and impart fluidity to the

membranes due to the presence of polyunsaturation in the acyl-chains which decrease the melting point giving the membrane its liquid-like characteristics (4). These fatty-acids are also precursors to a group of molecules called 'eicosanoids' -- a family of hormone-like molecules including prostaglandins, leukotrienes, and thromboxanes that are secondary messengers, regulators of endocrine action in tissues and are involved in the management of muscle contractions, coagulation, and immunity (5,6). A disruption in eicosanoid synthesis will have severe effects such as decreased immune response and inefficient coagulation (4). A depletion of available eicosanoid precursors (arachidonic and eicosapentaenoic acid) can therefore have detrimental effects on the body.

A significant threat to polyunsaturated fatty-acids is free radicals -- molecules with unpaired electrons (7). Some commonly encountered sources of free radicals are environmental pollutants such as pesticides, tobacco smoke, solvents, anesthetics, chemotherapeutic agents, and photo-chemical air pollutants. Tobacco smoke contains radical species which can injure cells and cause peroxidative damage which may lead to coronary heart disease (5). Each puff from a cigarette may contain as many as 10^{15} free radicals in the gas phase and 10^{14} free radicals in the tar

(particulate) phase (5). These free radicals are highly reactive and can be sources of tremendous damage within the body. Some free radicals, such as the extremely reactive hydroxyl radical ($OH^{\bullet}$), can react very quickly with biological molecules, severely altering their structures. Free radicals are thought to be a leading cause of aging and cancer because of their ability to transform the physical and chemical characteristics of numerous biological molecules ranging from DNA to lipids. Polyunsaturated fatty-acid acyl-chains are more susceptible to oxidative damage than fatty-acids with zero, one, or two double bonds (4). This is due to the 'methylene separated sites of unsaturation' pattern discussed earlier. The abstraction of a hydrogen from these methylene groups is aided by the double bond on either side of the group allowing for stabilization by electron delocalization.

Damage to the cell membrane, regardless of free radical source, can cause increased membrane permeability which can, in turn, cause damage to ion gradients, secretory functions, and metabolic processes (7). Peroxidative damage can also affect the fluidity of the cell membrane by allowing for the formation of bonds between the fatty-acid acyl-chains causing the formation of a more globular-like membrane rather than a fluid one (4). Once a membrane lipid has been

peroxidized it can lead to further peroxidation of other lipids as well as membrane proteins in what is termed propagation (4).

The radical species found in cigarette smoke can be divided into two categories; long-lived quinone-semiquinone radicals and short-lived reactive carbon- and oxygen-centered peroxy radicals (5). The long-lived quinone-semiquinone radical species (Figure 3) are produced during combustion by the oxidation of polycyclic aromatic hydrocarbons. The semiquinone radical ($Q^{\bullet}$) can reduce oxygen to superoxide (O_2^{-}) and hydrogen peroxide (H_2O_2). It can also cause the production of hydroxyl radicals ($OH^{\bullet}$) by way of the following mechanism:

$$O_2 + Q^{\bullet} \rightarrow O_2^{-} + Q$$

$$O_2^{-} + Q^{\bullet} + 2H^{+} \rightarrow H_2O_2 + Q$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{-} + OH^{\bullet} + Fe^{3+}$$

Figure 3. Examples of a quinone (Q) and a semiquinone radical ($Q^{\bullet}$).

The short-lived carbon- and oxygen-centered radicals have lifetimes of less than one second. However, because production of these radicals continues as the smoke ages, the radicals have an apparent lifetime of more than five minutes. Hydroxyl radicals can also be produced through the following mechanisms:

$NO + H_2O_2 \rightarrow HNO_2 + OH^{\bullet}$ $NO_2 + H_2O_2 \rightarrow HNO_3 + OH^{\bullet}$

The hydrogen peroxide in these mechanisms is either produced during the production of the long-lived radicals or can originate from pulmonary macrophages and neutrophils as part of their normal function (5).

The peroxidation of fatty-acid acyl-chains can be initiated by any molecule that is reactive enough to remove a hydrogen atom from a methylene ($-CH_2-$) group (4). The hydroxyl radical produced in cigarette smoke is capable of doing this and the abstraction of the hydrogen is aided by the presence of double bonds in the acyl-chains which weaken the C-H bonds on the α-carbons allowing for the easy removal of the hydrogen. The newly-formed carbon radical is stabilized by forming a conjugated diene which can then undergo further reactions with neighboring side-chains to propagate the chain reaction (Figure 4) (4).

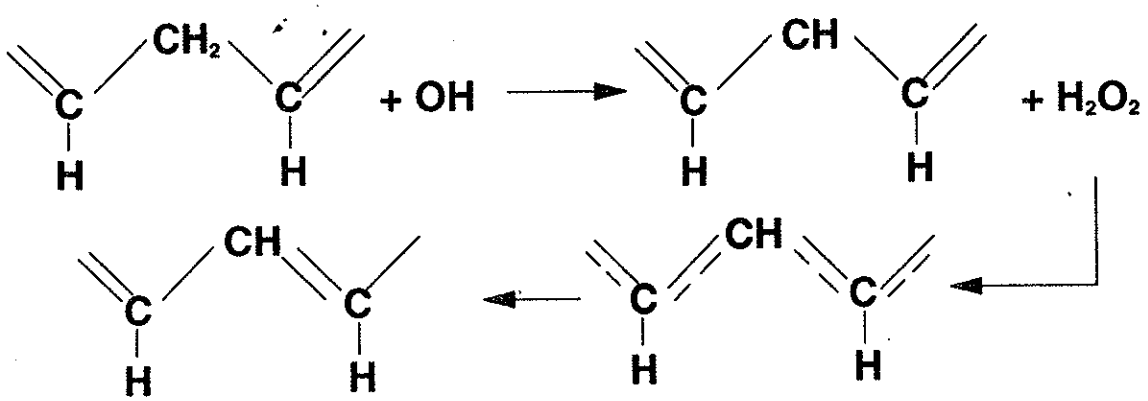


Figure 4. The peroxidation of fatty-acid side-chains.

Under aerobic conditions, the conjugated dienes produced above can react with O_2 to produce peroxyl radicals ($LOO^{\bullet}$). These peroxyl radicals are reactive enough to remove a hydrogen atom from a neighboring fatty-acid side-chain, forming a new free-radical and thus propagating the radical reaction through the cell-membrane (4). Because electrons are more stable when they are paired, the free-radicals will combine with other free-radicals to form pairs in what is called the termination step. Termination can occur in several ways following the peroxidaton of fatty-acid side-chains (8):

$$L^{\bullet} + L^{\bullet} \rightarrow LL$$

$$LO_2^{\bullet} + LO_2^{\bullet} \rightarrow LOOL + O_2$$

$$LO_2^{\bullet} + L^{\bullet} \rightarrow LOOL$$

The peroxidation of lipids, such as eicosapentaenoic acid, results in an alteration of the 'methylene-separated sites of unsaturation' pattern which prevents the fatty-acids from being used as precursors for eicosanoids. The free-radicals attack on fatty-acids also allows for the formation of cross-linkages between fatty-acids significantly altering the characteristics of the cell-membrane (8).

We assumed that the destruction of endogenous eicosapentaenoic acid in the cell membrane will generate an increased demand for synthesized eicosapentaenoic acid. This requirement may be met by the synthesis of eicosapentaenoic acid from linolenic acid. Therefore, when the eicosapentaenoic acid level in the body does not meet the body's demand, such as when the fatty-acids are altered by free radical attack, an increased rate of metabolism will result. Conversely, an increased rate of fatty-acid synthesis may signify the destruction of dietary fatty-acids. Therefore, we hypothesized that the free-radical induced destruction of fatty-acids would be evidenced by an increased rate of eicosapentaenoic acid synthesis in smokers.

Materials and Methods:

The goal of this study was to examine the possibility of tobacco smoke-induced free-radical attack on fatty-acids. This was done by examining the synthetic rate of n3 fatty-acids in both smokers and nonsmokers and our hypothesis was that free-radical attack on fatty-acids would lead to an increased demand for fatty-acids within the body and therefore an increased synthetic rate. Through the use of a deuterium tracer, the synthetic rates of n3 fatty-acids were determined in a group of smokers and a group of nonsmokers.

Study subjects

The study included 18 volunteers. The subjects were divided into two groups; 8 nonsmokers and 10 smokers. Group I (nonsmokers) consisted of 4 females and 4 males with an average age of 28.9 ± 3.0 years (range 20-44 years). Group II (smokers) consisted of 5 females and 5 males with an average age of 39.6 ± 5.1 years (range 29-60). The average ages were determined from the ages of the male volunteers because ages were not available for the female volunteers (I believe that NIAAA did not provide me with this information in order to prevent me from publishing this research on my own).

Volunteers were given a diet of known composition for

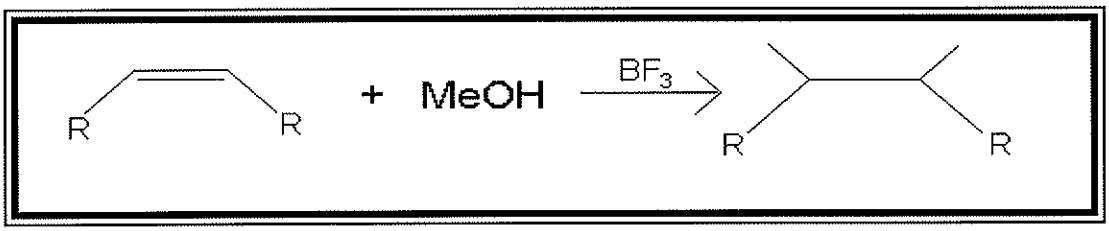
three weeks prior to plasma collection. The diets were specifically composed according to our assumption (fatty-acid synthesis only occurs when a deficiency is present) in order to arrest the synthesis of the fatty-acids studied. All appropriate precursors were provided as part of the diet. At t=0 hr, the volunteers were given a bolus (single) dose of C18:3n3 labeled with deuterium (d_5-labeled fatty-acids, *C18:3n3). The deuterium label served as a tracer which allowed us to observe the fatty-acids as they entered and exited specific fatty-acid pools. Plasma samples were obtained from the patients at t=0 hr, 8 hr, 24 hr, 48 hr, 72 hr, 96 hr, and 168 hr. Samples were stored at -80°C for further study.

Total lipid extraction

One mL BHT-MeOH reagent (3 µg 2,6-di-tert-butyl-cresol per mL methanol), 1 mL whole plasma, 1 µg 23:0 internal standard, 2 mL chloroform, and 0.6 mL water were mixed in a 25 mL centrifuge tube. The tube was purged with nitrogen and vortexed for 1 minute. Following the appearance of a single phase mixture, 1 mL chloroform and 1 mL water were added to the tubes and the mixture was vortexed for 1 minute. The tubes were centrifuged at 3000 RPM for 10 minutes or until two distinct liquid phases divided by a

solid phase appeared (sometimes requiring several hours). The organic layer was removed and the sample was re-extracted once. Extracts were stored under nitrogen at -80°C until transmethylation could proceed.

Transmethylation of lipid extracts



Extracted lipid samples were completely dried with nitrogen. Once the samples were dry, 1.25 mL reagent methanol, 0.75 mL n-hexane, and 1.0 mL 14% w/v BF_3-methanol reagent were added to the sample. The solutions were covered with a nitrogen blanket and the tubes were sealed. Samples were vortexed for 1 minute and were placed in a heating block at 100°C for 30 minutes. Samples were removed from the heating block, vortexed for 1 minute, and returned to the heating block for an additional 90 minutes. Once the samples had cooled to room temperature, 4 mL cold n-hexane and 3 mL cold water were added to the samples which were subsequently vortexed for 1 minute. Once a bilayer formed, the organics were removed and were placed in a 13 x 100 mm

test tube. The samples were extracted a second time with 3 mL n-hexane and the extracts were pooled. Sample volumes were reduced to approximately 0.1 mL with a stream of nitrogen and the samples were transferred to autosampler 'V-vials' for GC analysis.

Gas Chromatographic analysis of lipid extracts

A Hewlett-Packard 5890 gas chromatograph with a flame ionization detector and 7673A autosampler/injector with refrigerated multiple sample vial tray was used in the analysis. The column was a J & W DB-FFAP fused silica capillary column (30 meter x 0.25 m x 0.25 µm film thickness). GC conditions can be seen in Figure 5.

Original Temp	130°C-175°C	175°C-210°C	210°C-245°C
130°C	4°C per min.	1°C per min.	30°C per min.

Figure 5. GC conditions.

The injector and detector temperatures were held constant at 250°C.

GC/MS analysis of lipid extracts

The d_5-labeled fatty-acid concentrations of each patient's plasma at each time interval was determined by GC/MS. This work was performed prior to my joining this project (9).

Determination of fractional synthetic rate

The amount of d_5-labeled fatty-acid incorporated into the plasma was calculated using a trapezoidal equation to approximate the area under the hypothetical curve of the concentrations of d_5-labeled substrates versus time over 168 hours (Figure 6).

$$X_{\mu g/ml} = 4 \times ([\text{Time } 0] + [\text{Time } 8]) + 8 \times ([\text{Time } 8] + [\text{Time } 24]) + 12 \times ([\text{Time } 24] + [\text{Time } 48]) + 12 \times ([\text{Time } 48] + [\text{Time } 72]) + 12 \times ([\text{Time } 72] + [\text{Time } 96]) + 36 \times ([\text{Time } 96] + [\text{Time } 168])$$

Figure 6. Trapezoidal equation used in the determination of plasma incorporation of d_5-labeled fatty-acids (9).

This number was then multiplied by the plasma volume in order to determine the total amount of incorporated isotope. The specific activity (isotopic enrichment of the pool) of each fatty-acid at each time point was determined by dividing the concentration of d_5-labeled fatty-acid in the plasma by the concentration of unlabeled fatty-acid in the plasma. The use of specific activity was important because the pools were of different sizes. The fractional synthetic rates (FSR) for each of the ω3 fatty-acids as well as for the overall metabolism (18:3n3 to 22:6n3) were determined by dividing the average of the change in the specific activity

of the product pool by the average of the change in the specific activity of the precursor pool for each patient at each time point. In the synthesis of 20:5n3 from 18:3n3, 20:5n3 would be the product pool and 18:3n3 would be considered the precursor pool.

Results:

The deuterium enrichments of 18:3n3, 20:5n3, 22:5n3, and 22:6n3 were compared between both study groups over time and were found to be consistent between the smokers and nonsmokers through the entire 168-hour study period. These results can be seen in Figures 7-10.

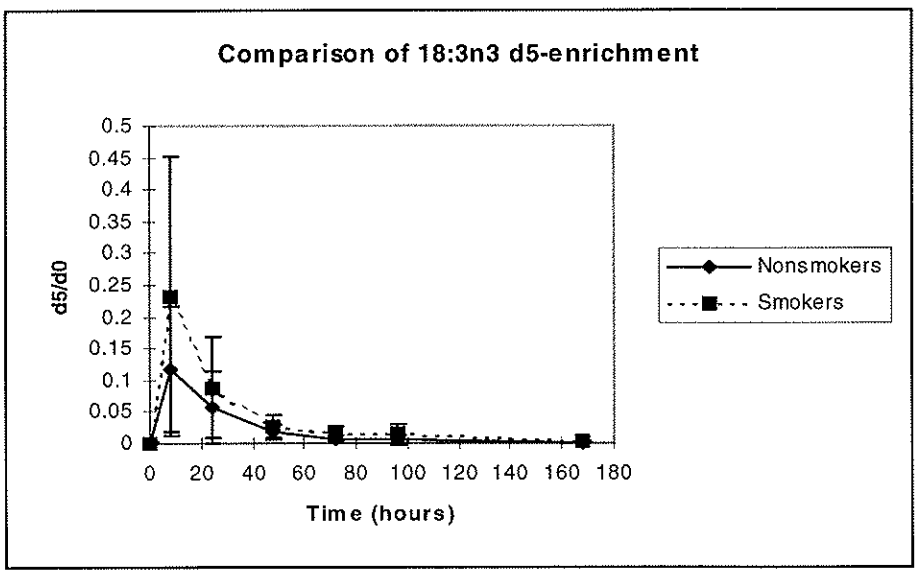


Figure 7. The comparison of d5-enrichment in 18:3n3 between smokers and nonsmokers.

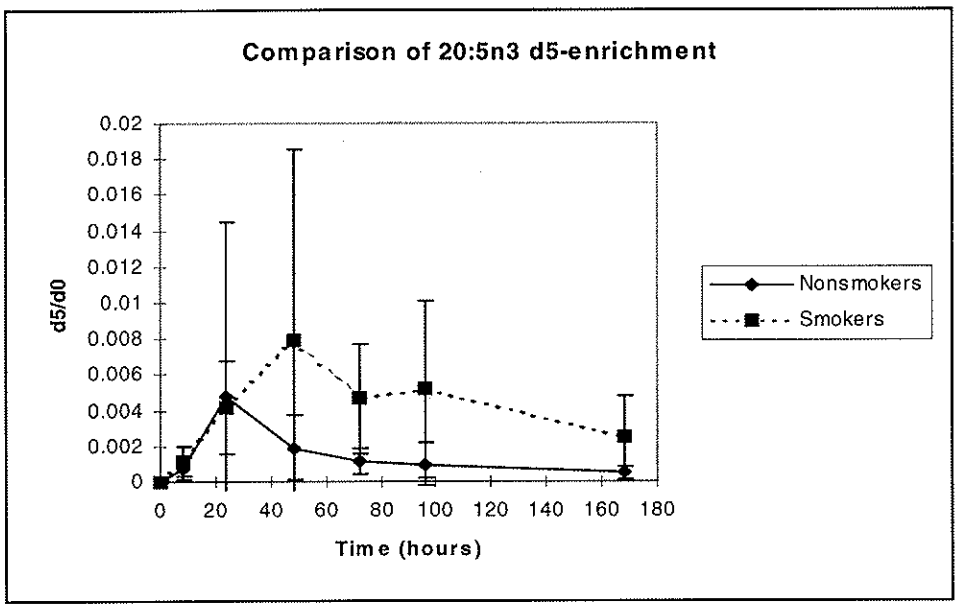


Figure 8. The comparison of d5-enrichment in 20:5n3 between smokers and nonsmokers.

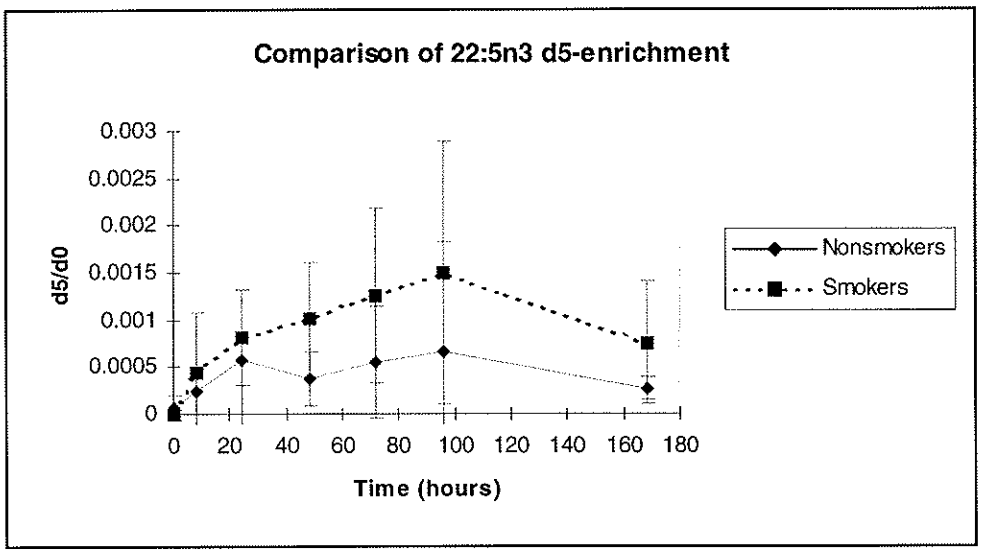


Figure 9. The comparison of d5-enrichment in 22:5n3 between smokers and nonsmokers.

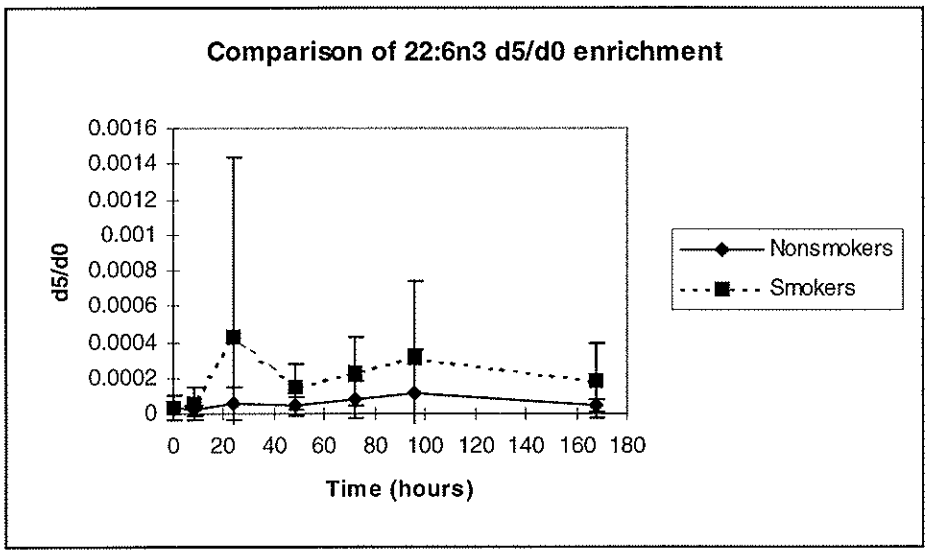


Figure 10. The comparison of d5-enrichment in 22:6n3 between smokers and nonsmokers.

Figure 11 shows that a significant difference was found between the eicosapentanoic acid synthesis of smokers and that of nonsmokers. The fractional synthetic rates (FSR) for the conversion of linolenic acid to eicosapentanoic acid were similar between 0 and 48 hours. At 72 hours, the FSRs of the smokers began to increase (0.341 at 72 hrs, 0.595 at 96 hrs, 1.23 at 168 hrs) while the FSRs of the nonsmokers remained fairly constant (0.294 at 72 hrs, 0.266 at 96 hrs, 0.462 at 168 hrs).

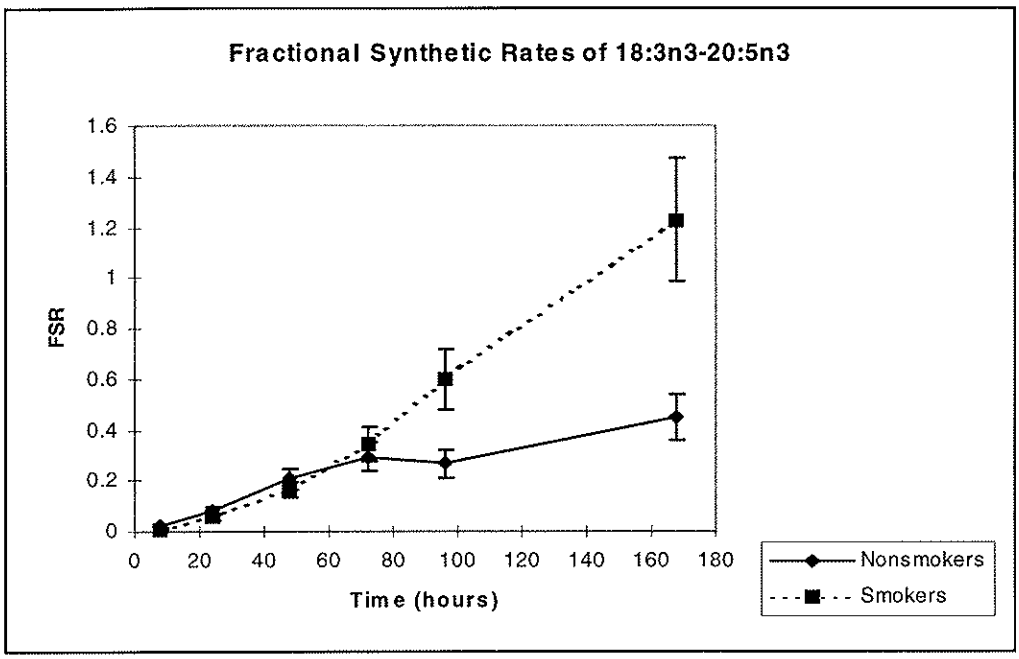


Figure 11. Fractional synthetic rates of 18:3n3-20:5n3 for smokers and nonsmokers.

Nonsmokers showed slightly higher FSRs for the conversion of 20:5n3 to 22:5n3 but the changes in rate were of the same magnitude for both the smokers and the nonsmokers (Figure 12).

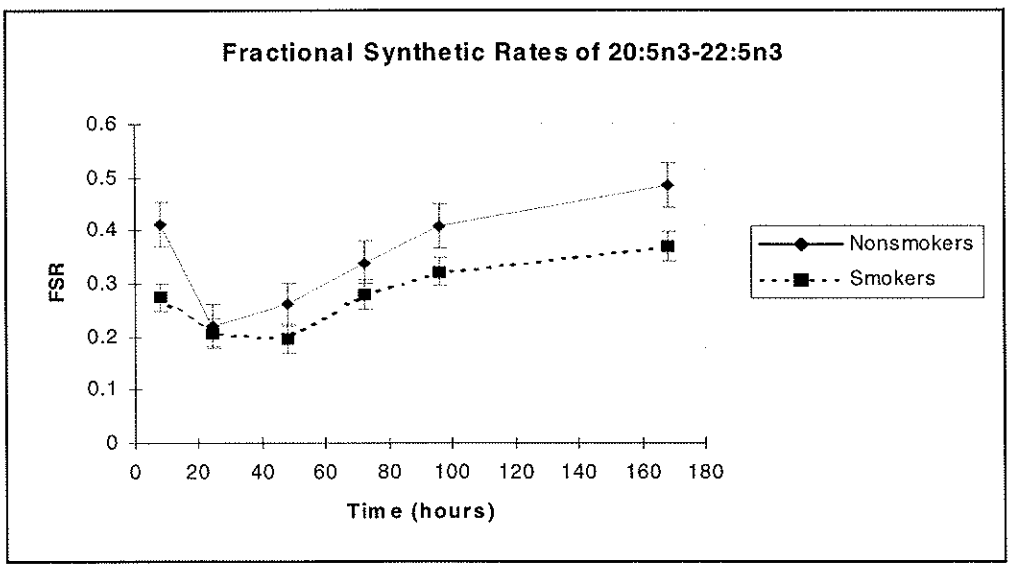


Figure 12. Fractional synthetic rates of 20:5n3-22:5n3 for smokers and nonsmokers.

The same consistency in rate changes was seen in the conversion of 22:5n3 to 22:6n3 (Figure 13). Here, however, the smokers had slightly elevated rates compared to those of nonsmokers.

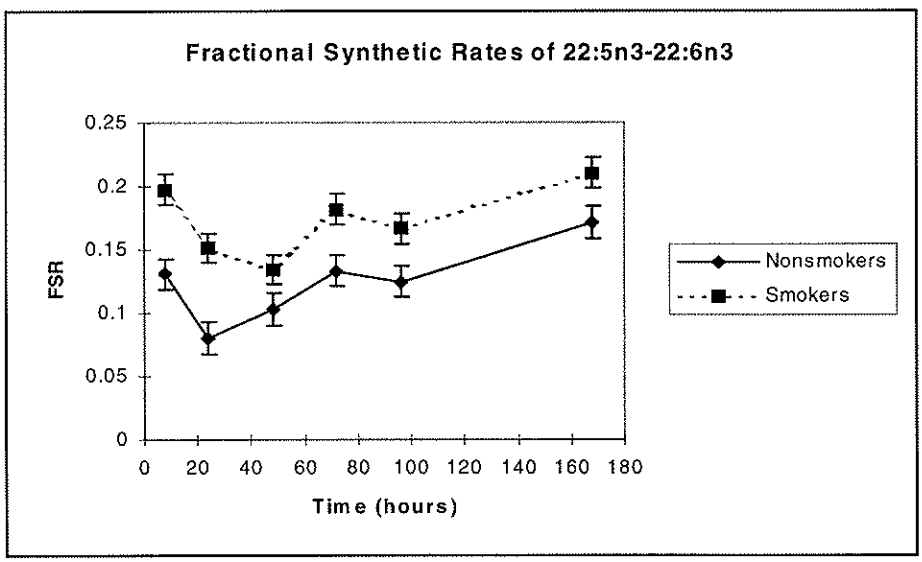


Figure 13. Fractional synthetic rates of 22:5n3-22:6n3 for smokers and nonsmokers.

Figure 14 shows that smokers had an increased rate for the overall conversion of linolenic acid to the final ω-3 fatty-acid product, 22:6n3 (docosahexanoic acid).

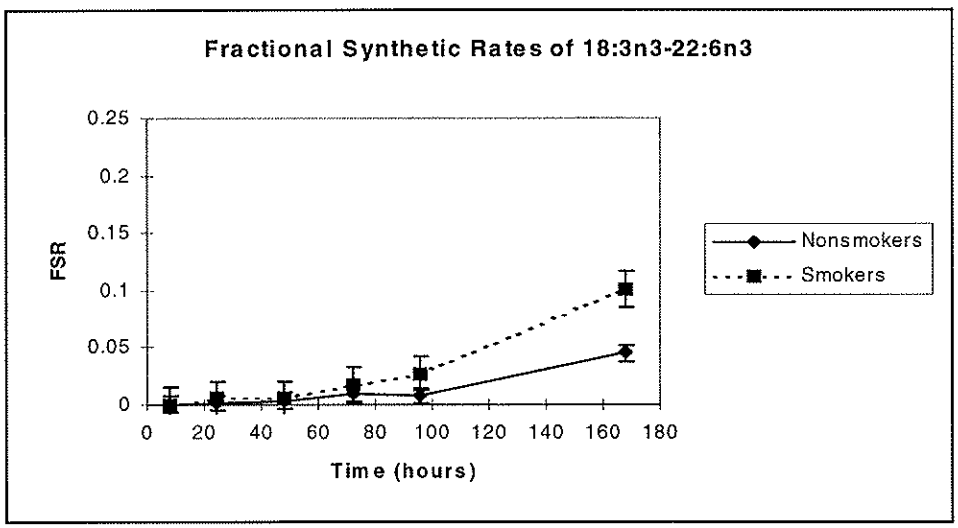


Figure 14. Fractional synthetic rates of 18:3n3-22:6n3 for smokers and nonsmokers.

Discussion:

Previous studies have shown that cigarette smoke can induce peroxidation of lipids *in vitro* (1). We can infer from the results of our study that cigarette smoke may be involved in the peroxidation of lipids *in vivo*. Figures 7-10 show that the pool-labeling with d_5 was consistent throughout the study. Therefore, any differences seen in the calculated fractional synthetic rates in Figures 11-14 can be attributed to a change in metabolic rate.

The increased rate of eicosapentanoic acid synthesis in smokers (18:3n3-20:5n3, Figure 11) suggests destruction of eicosapentanoic acid (20:5n3). Once the 20:5n3 is peroxidized, it can no longer be used as a precursor for vital molecules such as eicosanoids and it may form cross-links with other fatty-acids in the cell-membrane giving it a more globular characteristic. We assumed that this deficiency would cause the body to increase production of eicosapentanoic acid which would in turn be interpreted as an increase in the fractional synthetic rate for the fatty-acid.

The conversion of 20:5n3 to 22:5n3 (Figure 12) showed similar fluctuations in rate for both the smokers and nonsmokers throughout the 168 hour test period. The increased rate of C22:5n3 synthesis seen with the nonsmokers did not impact greatly on the expected result seen in Figure 14 (18:3n3-22:6n3). The smokers showed an increased synthetic rate for this synthesis

(18:3n3-22:6n3) as well and this rate is most likely due to the increased rate of conversion to 20:5n3.

The conversion of 22:5n3 to 22:6n3 (Figure 13) showed similar fluctuations as well. However, here the smokers had a slightly increased synthetic rate throughout the entire 168 hour test period. When the FSRs for the whole synthetic chain are observed (18:3n3-22:6n3, Figure 14), the smokers have an overall increased rate of synthesis compared to that of nonsmokers. This again is most likely due to the increased rate of synthesis seen in the production of eicosapentanoic acid.

Conclusion:

The results of this study indicate that cigarette smoke may have a significant effect on endogenous fatty-acids. The differences in fatty-acid synthetic rate between smokers and nonsmokers demonstrate that endogenous fatty-acids in the presence of cigarette smoke may be altered in such a manner that they become ineffective in the body. The discrepancy seen between smokers and nonsmokers may be due to free-radical attack on endogenous fatty-acids generating the need for fatty-acid synthesis within the body and thus an increased synthetic rate. The differences in synthetic rate may also be due to prior destruction or depletion of fatty-acid stores in the smokers' bodies necessitating increased synthetic rates regardless of free-radical intake. Repeated studies with more patients are necessary in order to establish the accuracy of this study. Further studies are also required in order to determine the extent of smoke-induced lipid peroxidation as well as a possible mechanism for the free-radical attack.

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