Journal of Biochemistry and Molecular Biology, Vol. 37, No. 6, November 2004, pp. 749-752

JOURNAL OF Biochemistry and Molecular Biology

Short communication

Lipid Peroxidation and the Thiobarbituric Acid Assay: Standardization of the Assay When Using Saturated and Unsaturated Fatty Acids

Leonard T. Rael[†,‡], Gregory W. Thomas[†,‡], Michael L. Craun[†], C. Gerald Curtis[§], Raphael Bar-Or[†,‡] and David Bar-Or[†,‡,*]

[†]Department of Trauma Research and Trauma Services, Swedish Medical Center, 501 E. Hampden Avenue, Englewood, CO 80113, USA

[‡]DMI BioSciences Inc., 3601 S. Clarkson St. #420, Englewood, CO 80110, USA

[§]Bowman Research (U.K.), Ltd., Imperial House, Imperial Park, Celtic Lakes, Newport, Gwent, NP10 9UH, United Kingdom

Received 27 April 2004, Accepted 3 June 2004

Saturated fatty acids are less vulnerable to lipid peroxidation than their unsaturated counterparts. In this investigation, individual fatty acids of the C_{16}, C_{18} and C_{20} families were subjected to the thiobarbituric (TBA) assay. These fatty acids were chosen based on their degree of saturation and configuration of double bonds. Interestingly, an assay threshold was reached where increasing the fatty acid concentration resulted in no additional decrease in the TBARS concentrations. Therefore, the linear range of TBARS inhibition was determined for fatty acids in the C_{16} and C_{20} families. The rate of TBARS inhibition was greater for the saturated than for unsaturated fatty acids, as measured from the slope of the linear range. These findings demonstrate the need to standardize the TBARS assay using multiple fatty acid concentrations when using this assay for measuring *in vitro* lipid peroxidation.

Keywords: Assay standardization, Degree of saturation, Fatty acids, Lipid peroxidation,TBA assay

Introduction

The debate over the beneficial effects of saturated versus unsaturated fatty acids has been a topic of research among the world's leading nutritional experts. A diet containing fats of the unsaturated variety has been shown to be beneficial in the prevention of atherosclerosis and coronary heart disease (Wolfram, 2003). Long-term diets containing monounsaturated fatty acids have been shown to reduce platelet aggregation and decrease plasma LDL-cholesterol levels (Smith *et al.*, 2003). Fish oil, which is high in long chain omega-3 polyunsaturated fatty acids, prevents endothelial activation by inhibiting the expression of adhesion molecules and improves vascular nitric oxide production (Christon, 2003). Saturated fatty acids undergo less peroxidation than their unsaturated counterparts. Indeed, supplementation with polyunsaturated as opposed to saturated fatty acids results in a statistically significant increase in lipid peroxidation in the plasma and liver (Song *et al.*, 2000; Song and Miyazawa, 2001; Shin, 2003). Also, age appears to be a risk factor in diets containing polyunsaturated fats. Oxidative damage to DNA in bone marrow was recorded in aged, but not young rats when a polyunsaturated diet was employed (Umegaki *et al.*, 2001).

Many oil formulations contain a wide variety of fatty acids of various carbon chain lengths and degrees of saturation. In the current study, our interest was in assessing the lipid peroxidation of fatty acids, as measured by the thiobarbituric acid (TBA) assay. Individual fatty acids of the C_{16}, C_{18} and C_{20} families, containing varying degrees of saturation (i.e. amount of double bonds), were assayed in a system containing copper and ascorbate. A plateau was reached where no additional decrease in the TBA-reactive species (TBARS) was observed, regardless of increases in the fatty acid concentrations. This limitation to the TBA assay suggests the need for a standardized assay using multiple concentrations of a fatty acid prior to drawing any conclusions on *in vitro* lipid peroxidation.

Materials and Methods

TBARS assay All reagents were purchased from Sigma-Aldrich (St. Louis, USA). The thiobarbituric acid (TBA) assay was used to assess lipid peroxidation using the method of Bar-Or *et al*., (2001), with a few modifications. All fatty acids were initially prepared as stock solutions in acetone to facilitate solvation. In the final reaction mixture, individual fatty acid concentrations ranged from 0-0.2

*To whom correspondence should be addressed.
Tel: 1-303 -788-4089; Fax: 1-303-788-4064
E-mail: dbaror@dmibio.com

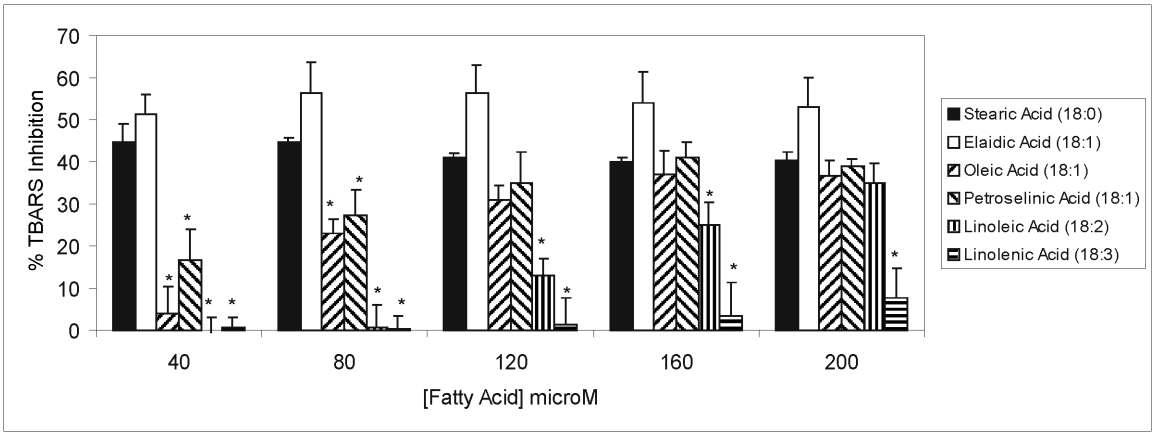

Fig. 1. TBA assay results for C18 fatty acids. Data is expressed as % TBARS inhibition compared to a positive control (error bars expressed as ± SD). Asterisks (*) indicate significantly less than percent (%) TBARS inhibition caused by saturated fatty acid (p<0.05).

mM, while the acetone concentration was 0.5% (v/v). Samples were pre-incubated for 15 min with a fatty acid or 0.5% acetone (v/v; positive control) and 0.01 mM $CuCl_2$ in 20 mM phosphate buffer (pH 7.4). The reaction was started by the addition of 0.5 mM ascorbate and 1.9 mM deoxyribose, and incubated for 1 hour at 37°C. 10 g/L TBA in 50 mM NaOH and concentrated acetic acid were added to the incubation mixture, boiled in water for 15 min and the thiobarbituric acid-reactive species (TBARS) quantified by spectrophotometry at 532 nm. The TBARS in the fatty acid-containing samples were compared with the positive controls containing 0.5% (v/v) acetone.

Statistical analysis Each data point is the average of triplicate measurements, with each individual experiment performed in duplicate. Linearity was determined using Microsoft Excel. Data were compared using paired, two-tailed Student's *t*-tests, with a value of $p < 0.05$ used to indicate a statistical significance.

Results and Discussion

Six fatty acids from the C_{18} family were subjected to the thiobarbituric (TBA) assay to elucidate how fatty acid characteristics, such as monounsaturation and polyunsaturation as well as double bond configurations (i.e. *cis*- versus *trans*-), affect the assay. As seen in Fig. 1, a threshold of 40-60% TBA-reactive species (TBARS) inhibition seems to be the point where the assay reaches a plateau. The assay plateau was studied further by subjecting fatty acids from the C_{16} and C_{20} families to the TBA assay. Fig. 2A shows that palmitic acid (16:0) was approximately 12 times more effective at decreasing TBARS formation compared to palmitoleic acid (*cis*-9-16:1), as measured from the slope of the linear portion of the reaction. For the C_{20} family, the same trend was observed (Fig. 2B). The saturated fatty acid, arachidic acid (20:0), was 3 times more effective at preventing the formation of TBARS than its unsaturated fatty acid counterpart, arachidonic acid (*cis*-5,8,11,14-20:4). The linearity of the reaction for the C_{20} fatty acids was better than that for the C_{16} fatty acids. Fatty acids were found not to interfere with the binding between TBA and malondialdehyde (data not shown), as shown by the addition of fatty acids to the positive control during the TBA addition step.

The purpose of this study was to standardize the thiobarbituric acid (TBA) assay by measuring the *in vitro* lipid peroxidation from individual fatty acids of the C_{16}, C_{18} and C_{20} families. TBA reacts to give multiple biomolecular breakdown products that have undergone free radical attack to form TBA reactive species (TBARS). The TBA assay is not specific for malondialdehyde (MDA), one of many breakdown products of degraded fatty acids. The nonspecificity probably results from the acid-heating step of the TBA assay that causes the formation of artifactual TBA/MDA-like derivatives (Liu *et al.*, 1997). However, the TBA assay is more than adequate for our purposes, as our only interest was in measuring the *in vitro* ability of fatty acids to prevent the production of TBARS, whether MDA or an MDA-like derivative.

To assess the *in vitro* free-radical scavenging ability of each fatty acid, the standardization of the TBA assay was required. This was achieved by choosing fatty acid concentrations that fell within the linear portion of the fatty acid concentration versus percent TBARS inhibition plot. A regression line plotted through the origin demonstrated the dose-response TBARS inhibition for fatty acids in the C_{16} and C_{20} groups. The TBARS inhibition efficiency for each fatty acid was equal to the slope (units = % TBARS inhibition per μM fatty acid) of the regression line, which is useful in the determination of the fatty acid concentration necessary to achieve a given percentage of TBARS inhibition. For example, arachidic acid (slope = 0.5515) gave 50% TBARS inhibition at a concentration of 90.7 μM under our assay conditions. Of course, obtaining 100% inhibition would be impossible using this *in vitro* assay, but can still be used as an initial step in determining an adequate fatty acid level in a controlled *in vitro* environment.

The important finding of this study was the need to standardize the TBA assay, which can be achieved by

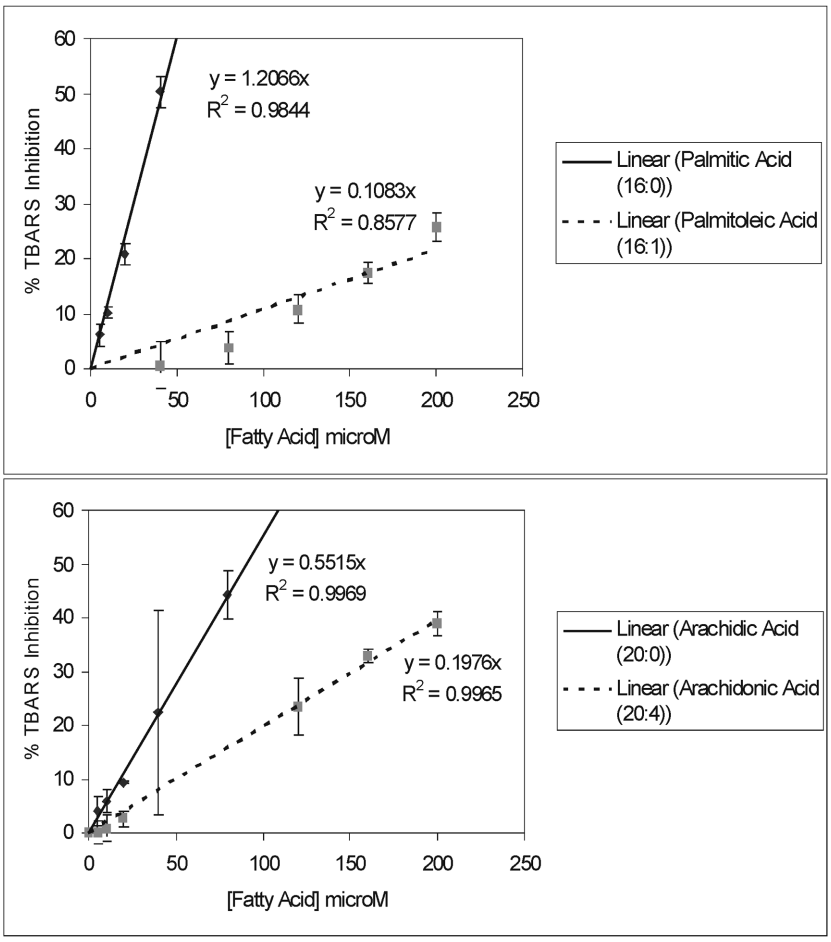

Fig. 2. TBA assay results for C16 (A) and C20 (B) fatty acids. Data is expressed as % TBARS inhibition compared to a positive control (error bars expressed as ± SD). Fatty acid concentrations were chosen that did not exceed the plateau of the TBA assay (approximately 50% TBARS inhibition). Linearity was determined using a linear regression line through the origin (Microsoft Excel program).

comparing the TBARS inhibition efficiency of each fatty acid, rather than choosing an arbitrary fatty acid concentration. Therefore, caution is advocated in the interpretation of *in vitro* lipid peroxidation, using only the TBA assay without standardization, when fatty acids are present in the assayed solution. More importantly, the nature of the fatty acid content, saturated versus unsaturated fatty acids, determines the degree of inhibition of TBARS production. Although the TBA assay lacks specificity, and is limited by interfering substances, it can still be a useful tool for assessing lipid peroxidation in a well-defined *in vitro* setting. Proper assay standardization and an understanding of the limits of the TBA assay are essential for comparison of the *in vitro* free-radical scavenging properties of various fatty acids.

Acknowledgment This work was supported by the Trauma Research Department, Swedish Medical Center (Englewood, USA) and DMI BioSciences Inc. (Englewood, USA).

References

Bar-Or, D., Rael, L. T., Lau, E. P., Rao, N. K., Thomas, G. W., Winkler, J. V., Yukl, R. L., Kingston, R. G. and Curtis, C. G. (2001) An analog of the human albumin N-terminus (Asp-Ala-His-Lys) prevents formation of copper-induced reactive oxygen species. *Biochem. Biophys. Res. Commun.* **284**, 856-862.

Christon, R. A. (2003) Mechanisms of action of dietary fatty acids in regulating the activation of vascular endothelial cells during atherogenesis. *Nutr. Rev.* **61**, 272-279.

Liu, J., Yeo, H. C., Doniger, S. J. and Ames, B. N. (1997) Assay of aldehydes from lipid peroxidation: gas chromatograph mass spectrometry compared to thiobarbituric acid. *Anal. Biochem.* **245**, 161-166.

Shin, S. J. (2003) Vitamin E modulates radiation-induced oxidative damage in mice fed a high-lipid diet. *J. Biochem. Mol. Biol.* **36**, 190-195.

Smith, R. D., Kelly, C. N., Fielding, B. A., Hauton, D., Silva, K. D., Nydahl, M. C., Miller, G. J. and Williams, C. M. (2003)

Long-term monounsaturated fatty acid diets reduce platelet aggregation in healthy young subjects. *Br. J. Nutr.* **90**, 597-606.

Song, J. H., Fujimoto, K. and Miyazawa, T. (2000) Polyunsaturated (n-3) fatty acids susceptible to peroxidation are increased in plasma and tissue lipids of rats fed docosahexaenoic acid-containing oils. *J. Nutr.* **130**, 3028-3033.

Song, J. H. and Miyazawa, T. (2001) Enhanced level of n-3 fatty acid in membrane phospholipids induces lipid peroxidation in rats fed dietary docosahexaenoic acid oil. *Atherosclerosis* **155**, 9-18.

Umegaki, K., Hashimoto, M., Yamasaki, H., Fujii, Y., Yoshimura, M., Sugisawa, A. and Shinozuka, K. (2001) Docosahexaenoic acid supplementation-increased oxidative damage in bone marrow DNA in aged rats and its relation to antioxidant vitamins. *Free Radic. Res.* **34**, 427-435.

Wolfram, G. (2003) Dietary fatty acids and coronary heart disease. *Eur. J. Med. Res.* **8**, 321-324.