Biochimica et Biophysica Acta, 645 (1981) 17–23
Elsevier/North-Holland Biomedical Press

BBA 79272

A MODEL FOR THE EFFECT OF LIPID OXIDATION ON DIPHENYLHEXATRIENE FLUORESCENCE IN PHOSPHOLIPID VESICLES *

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(Received November 27th, 1980)

Key words: Lipid oxidation; Diphenylhexatriene; Fluorescence; Liposome

We have determined by means of a standard spectrophotometric assay that lipid oxidation occurred at a significant rate in large, multilamellar vesicles containing egg phosphatidylcholine under normal experimental conditions. We have also observed that the fluorescence intensity of the vesicle-associated probe, 1,6-diphenyl-1,3,5-hexatriene, decreased with time in vesicles containing such oxidizing lipids. The spectrophotometric data utilized to monitor lipid oxidation were found to fit an apparent first-order kinetic model. The loss of diphenylhexatriene fluorescence intensity in oxidizing liposomes was analyzed in terms of a first-order event superimposed (and thus presumably dependent) upon the ongoing formation of oxidized lipid. These and other data were used to conclude that the oxidation-induced loss of diphenylhexatriene fluorescence intensity was due to chemical modification of the fluorophore rather than to excited-state quenching or ground-state complex formation. Finally, the loss of fluorescence intensity in oxidizable membranes was found to alter drastically the 'microviscosity' parameter as derived from diphenylhexatriene fluorescence anisotropy and relative intensity measurements.

Introduction

As described by Klein [2], the relative absorbance of the conjugated-diene hydroperoxides formed in oxidizing lipids (maximum absorbance at 233 nm) can be used as a sensitive and convenient measure of oxidation in polyunsaturated lipids. We have used Klein's 'oxidation index' (the conjugated diene peak absorbance normalized to the concentration of the lipid, as determined by the absorbance at 215 nm) routinely as a test for oxidation in our unsaturated lipid stocks such as egg phosphatidylcholine.

While monitoring the fluorescence of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into vesicles containing egg phosphatidylcholine and cholesterol, we repeatedly observed anomalous decreases in the DPH fluorescence intensity. We did not observe this behavior in similar experiments [3] with the saturated lipid 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine mixed with cholesterol.

Here we present evidence that a correlation exists between the presence of lipid oxidation products and the reduction of DPH fluorescence intensity in egg phosphatidylcholine and egg phosphatidylcholine/cholesterol vesicles. Furthermore, we present simple kinetic models both for the rate of lipid oxidation in these membranes and for the rate of loss of DPH fluorescence intensity. The results suggest a mechanism for the loss of DPH intensity due to lipid oxidation.

The relevance of this study is primarily two-fold. First, there are now a great number of investigators employing the fluorescent probe DPH in order to relate the structure and function of membrane lipid

* A preliminary account of this work was presented at the Joint Meeting of the Biophysical Society and the Federation of American Societies for Experimental Biology, held June 1–5, 1980 in New Orleans, LA [1].

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Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

bilayers. For this, DPH has proven to be a useful and powerful tool, but some precautions must be taken in order to avoid its misuse [3–7]. The results presented here add to our knowledge of the proper use of this extremely powerful membrane probe. Second, the process of lipid or tissue oxidation has long been implicated in the etiology of a variety of degenerative diseases [8]. The mechanisms by which the highly unsaturated lipids of many cell types are protected from oxidation are by no means fully understood, but one of the most likely possibilities is that the fat-soluble vitamin E is involved (for review, see Ref. 9). Definition of the mechanisms of lipid oxidation and the secondary reactions caused by oxidation products, as well as the elucidation of how biological systems are normally protected from these events, should prove to be an important area for future research.

Materials and Methods

Egg phosphatidylcholine, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine and [4-^{14}C]cholesterol were prepared as described previously [3]. Zone-purified DPH was a gift from Y. Barenholz and M. Shinitzky. Large, multilamellar vesicles were formed as previously described [3] in 0.050 M KCl. The KCl solution was prepared from deionized, doubly glass-distilled, argon-saturated water and ultrapure KCl (Alfa-Ventron, Beverly, MA). Small, unilamellar vesicles were prepared by sonicating the large vesicles in a Heat Systems® Cup Horn® Sonicator, using a modification of the procedure previously described [10]. Instead of using a glass ampoule, the sample was sonicated in a 10 ml polycarbonate tube (Dupont Sorvall, Wilmington, DE) suspended approx. 5 mm above the surface of the horn. The use of polycarbonate greatly enhanced the transfer of sonic energy to the sample and, therefore, reduced the time to sample clarity (2 min for 2 ml of 5 mM egg phosphatidylcholine). However, the benefit of increased efficiency of sonication was, in the case of difficult-to-sonicate samples, counterbalanced by occasional contamination from the polycarbonate tube. In the present study, this was avoided by performing several 2-min sonications with only buffer in the tube. The release of contaminant was followed by monitoring of the ultraviolet spectrum of the sonicated buffer (λ_{max} of released material = 250 nm) until essentially no more was released. The tube could then be used for a short sonication of a lipid sample. However, very concentrated samples (>10 mM) or samples containing lipids with a long clarification time could sometimes not be clarified by this method without softening of the polycarbonate tube accompanied by further release of contaminants. All samples used here were prepared under conditions that avoided this complication.

Fluorescence intensity and anisotropy measurements were made on an SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL) with a thermally jacketed cell holder temperature-controlled by a Neslab RTE-8 Circulating Bath (Neslab, Portsmouth, NH). Using the SLM 4800, the intensity of light scattered from vesicle samples was always less than 0.1% of the fluorescence intensity. Fluorescence lifetimes were determined, using the SLM spectrofluorometer, by the phase and intensity modulation techniques [11]. Relative fluorescence intensity measurements were made in comparison with a standard of Rhodamine 6G in a sealed cuvette. As previously reported [6], it was unnecessary to correct the fluorescence anisotropy measurements for light-scattering-induced depolarization in egg phosphatidylcholine-containing samples.

The lipid oxidation index was measured as the ratio of optical densities at 233 and 215 nm in samples containing approx. 0.2 mM lipid in absolute ethanol as described by Klein [2]. For these measurements, aliquots of aqueous vesicle samples were extracted with chloroform/methanol by the procedure of Bligh and Dyer [12]; the lower (chloroform-rich) phase was evaporated to dryness under a stream of Argon, and then the lipid was resuspended in absolute ethanol. Although some samples contained DPH, we note that the small amounts used here did not interfere with the oxidation index measurement, nor did the cholesterol, which does not absorb significantly at 233 nm but does absorb at 215 nm with an extinction coefficient similar to that of the egg phosphatidylcholine. Optical density measurements were made using a GCA/McPherson Series 700 Spectrophotometer with a deuterium lamp and a slit width of 1 mm.

Results and Discussion

The loss of DPH fluorescence intensity in multilamellar vesicles has been monitored as a function of

time for several samples composed of egg phosphatidylcholine mixed with varying amounts (0–50 mol%) of cholesterol. Typical of the data for all these experiments are the curves shown in Fig. 1A for both egg phosphatidylcholine and 25 mol% cholesterol/egg phosphatidylcholine incubated at 25°C. Here the DPH (1 per 1000 lipids) was colyophilized with the lipids to allow complete uptake and maximum fluorescence intensity (F_0) at the time (0 h) of dispersion in 0.050 M KCl. Similar fluorescence intensity curves (not shown) for samples to which the DPH was added after dispersion showed that at 25°C, 24 h or more were required to achieve 90% of maximum intensity in large, multilamellar vesicles. By this time, as can be seen in Fig. 1A, the decrease in intensity of colyophilized DPH had become substantial. At times longer than 48 h after dispersion, the time-dependence of DPH relative fluorescence intensity was similar in samples prepared by either method. It should be noted that the relative fluorescence intensity of DPH remained constant (±5%) for at least 4 weeks at 25°C in multilamellar vesicles composed of a saturated lipid such as 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine. Thus, the DPH appeared to be inherently stable. We therefore sought to determine if the intensity de-

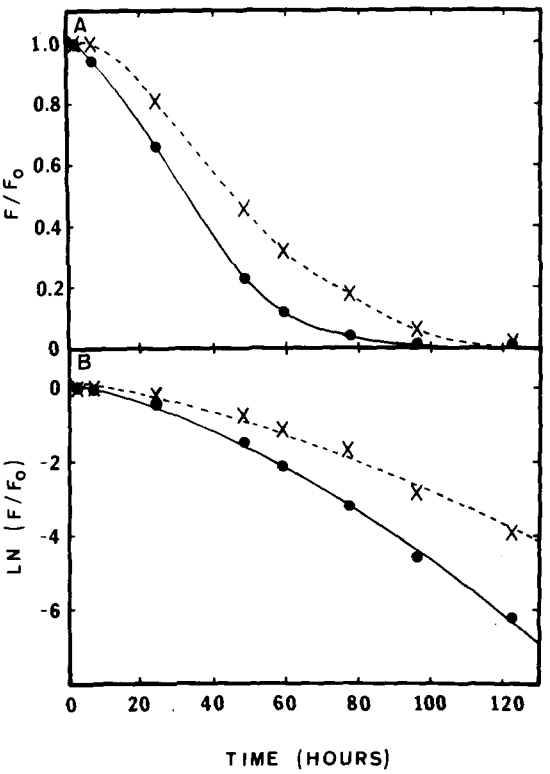

Fig. 1. A. Normalized DPH fluorescence intensity (F/F_0) vs. time for egg phosphatidylcholine (X- - - - -X) and 25 mol% cholesterol/egg phosphatidylcholine (•———•) multilamellar vesicles incubated at 25°C. DPH was colyophilized with the lipids to achieve complete uptake at the time (0 h) of dispersion in 0.050 M KCl. The curves are hand drawn through the data. B. Natural logarithm of the normalized DPH fluorescence intensity as a function of time for egg phosphatidylcholine (X- - - - -X) and 25 mol% cholesterol/egg phosphatidylcholine (•———•) multilamellar vesicles. The curves represent a best fit of the proposed model for DPH intensity loss to the data (see Eqn. 5 of text).

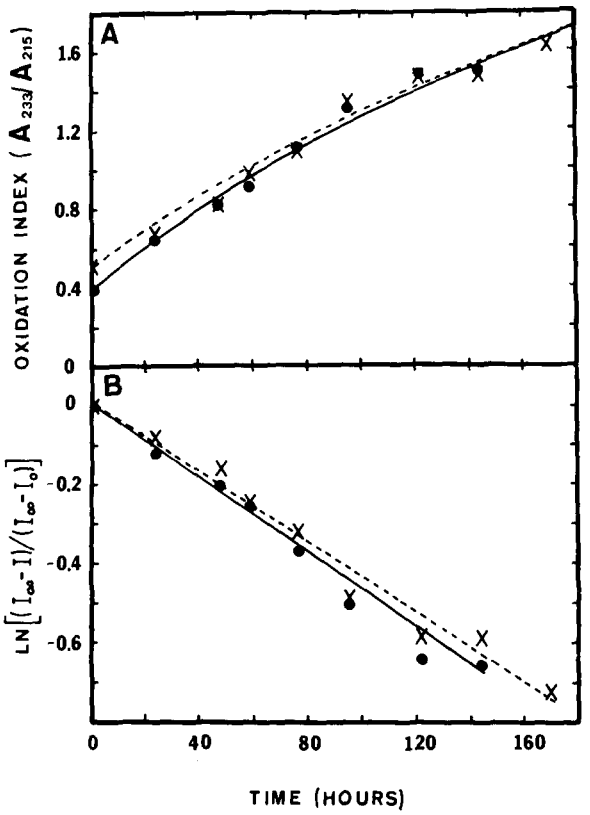

Fig. 2. A. Oxidation index plotted versus time for the egg phosphatidylcholine (X- - - - -X) and 25 mol% cholesterol/egg phosphatidylcholine (•———•) multilamellar vesicle samples used for the experiment depicted in Fig. 1. The curves are hand drawn through the data. B. $\mathrm{Ln}(I_\infty - I)/(I_\infty - I_0))$ versus time (see Eqn. 1 of text) for egg phosphatidylcholine (X- - - - -X) and 25 mol% cholesterol/egg phosphatidylcholine (•———•) multilamellar vesicles. The curves result from least-squares linear regression analysis of the data.

crease observed in egg phosphatidylcholine samples might be related to the greater susceptibility of this lipid to oxidation.

In Fig. 2A, we have plotted the spectrophotometrically determined oxidation indices for the same samples used for Fig. 1, measured at various times after dispersion of the lipids. A double reciprocal plot (not shown) of the data extrapolated to infinite time gave an oxidation index of 2.7 for both samples, which we used as I_∞ in later calculations. The initial oxidation index measurements in Fig. 2 were made 30 min after dispersion and showed considerably higher values (0.500 for egg phosphatidylcholine and 0.391 for 25% cholesterol/egg phosphatidylcholine, defined as I_0 in later calculations) than the oxidation index of our egg phosphatidylcholine stock solution (0.220). This difference presumably represents oxidation occurring during either lyophilization, sample dispersion, the extraction procedure described above (see Materials and Methods) or any combination of the above.

If we assume that the oxidation of the egg phosphatidylcholine is a simple, first-order process, either irreversible, or far from equilibrium, then it may be described by the expression: unoxidized lipid $\xrightarrow{k}$ oxidized lipid. Further, we will assume that the normalized oxidation index, I/I_∞, is equivalent to the oxidized fraction of potentially oxidizable lipid. With these assumptions, one derives:

$$\ln((I_\infty - I)/(I_\infty - I_0)) = -kt \qquad (1)$$

where I = oxidation index (A_{233}/A_{215}), I_0 = oxidation index at zero time (time of sample dispersion), and I_∞ = oxidation index at infinite time (extrapolated, see above).

Fig. 2B shows the oxidation index data from Fig. 2A transformed according to Eqn. 1. Based on Eqn. 1, least-squares linear regression analysis gave rate constants of $(4.44 \pm 0.27) \cdot 10^{-3}$ h^{-1} for the egg phosphatidylcholine data and $(4.75 \pm 0.58) \cdot 10^{-3}$ h^{-1} for the 25 mol% cholesterol/egg phosphatidylcholine data shown (these values will be used as the oxidation rate constants in later calculations). The log-linear fit of the data support the conclusion that the oxidation of the lipid (as monitored by the spectrophotometric assay for conjugated double bonds) obeyed pseudo-first-order kinetics.

It is apparent, however, that the loss of DPH fluorescence intensity in multilamellar vesicles composed of these oxidizing lipids did not fit such a simple kinetic scheme, as evidenced by the non-linearity of the logarithmically transformed data (Fig. 1B). In order to fit the loss of DPH fluorescence intensity to a model involving lipid oxidation, we made the following assumptions: (i) that the loss of fluorescence was directly related to the appearance of a modified form of DPH, DPH_N; (ii) that the rate of DPH_N formation was related to the fraction of oxidizable lipid oxidized (I/I_∞, in Eqn. 1); (iii) that the rate of DPH_N formation was first-order with respect to the concentration of fluorescent DPH, [DPH]. With these assumptions, one may write:

$$F(t)/F_0 = \frac{[\mathrm{DPH}]_0 - [\mathrm{DPH_N}]_t}{[\mathrm{DPH}]_0} \qquad (2)$$

and,

$$\frac{\mathrm{d}[\mathrm{DPH_N}]}{\mathrm{d}t} = k_1[\mathrm{DPH}]_t(I/I_\infty) \qquad (3)$$

where $[\mathrm{DPH}]_t$ and $[\mathrm{DPH_N}]_t$ represent the concentrations of fluorescent and modified DPH at time t and k_1 is the second-order rate constant for the modification of DPH in the presence of oxidized lipid. Solution of the differential equation in Eqn. 3 followed by substitution of Eqn. 2 into the result yields:

$$\ln(F/F_0) = (k_1/k)((I_0 - I_\infty)/I_\infty)e^{-kt} - k_1 t + L \qquad (4)$$

where k, I_0 and I_∞ are the parameters characterizing lipid oxidation, as described above, and L is a constant of integration.

Using the previously determined values for k, I_0 and I_∞, best fit values for k_1 (and its dependent parameter L) have been determined for these data sets in the following manner. Eqn. 4 can be rearranged to give a function linear in time:

$$-\ln(F/F_0) + (k_1/k)((I_0 - I_\infty)/I_\infty)e^{-kt} = k_1 t - L \qquad (5)$$

Using an initial estimate for k_1 in the left side of Eqn. 5 and substituting in previously determined values for k, I_∞, and I_0, along with experimental values for F/F_0, we have transformed our data into the pre-

dicted linear form. When the entire data set was thus transformed, the least-squares linear regression slope (of the transformed data versus time) was taken as the new estimate of k_1, and the process was repeated. This iterative procedure rapidly converged to best fit values of $k_1 = 0.087\ h^{-1}$, ($L = 16.15$) for the egg phosphatidylcholine data and $k_1 = 0.150\ h^{-1}$, ($L = 27.29$) for the 25 mol% cholesterol/egg phosphatidylcholine data of Fig. 1. The fit of the data to the resultant curves is shown in Fig. 1B.

The implication of this model for the time-dependent loss of DPH fluorescence intensity is that the conversion of DPH to a non-fluorescent product is a first-order process with respect to DPH concentration. In addition, the rate of fluorescence loss is also directly related to the amount of lipid oxidized. Since the amount of oxidized lipid increased during the experimental time course (see Fig. 2), the relative fluorescence intensity reflected the superposition of two first-order events leading to the complex time dependence shown in Fig. 1.

Our model predicts that if the level of oxidized lipid were constant, the intensity of DPH fluorescence should decrease in a simple, first-order manner. To test this prediction, DPH-labelled, egg phosphatidylcholine-multilamellar vesicles were incubated at 47°C until the DPH fluorescence had diminished to less than 1% of its original intensity (5 days). Then the sample was sonicated (see Materials and Methods) and fresh DPH was injected into the vesicle dispersion. In contrast to its rather slow uptake into multilamellar vesicles, DPH is incorporated into sonicated vesicles fairly rapidly. Thus, it was possible to record significant fluorescence intensity data within 5 h following the DPH addition. An oxidation index measurement was taken at this time which revealed that the sample was oxidized to such a great extent that the 233 nm peak (characteristic of conjugated-diene absorption) appeared to engulf the lower wavelength peak, producing a spectrum considerably different from those observed for the less oxidized samples of Fig. 2. On this basis, this egg phosphatidylcholine sample appeared to be essentially completely oxidized. The results of this experiment, plotted as the natural logarithm of the normalized fluorescence intensity (with F_0 determined by extrapolation), are shown in Fig. 3. As predicted, with the amount of oxidized lipid remaining nearly constant, the decrease in DPH fluorescence intensity obeys first-order kinetics as shown by the linear logarithmic fit. The rate constant, k_1, determined as minus the slope of the data in Fig. 3, was $0.024\ h^{-1}$ and was, therefore, of the same order of magnitude as that obtained from the data in Fig. 1B ($0.087\ h^{-1}$ for egg phosphatidylcholine). One possible explanation for the discrepancy between these values is the fact that the data in Fig. 1B were obtained using multilamellar vesicles while the results in Fig. 3 were of necessity obtained with sonicated vesicles.

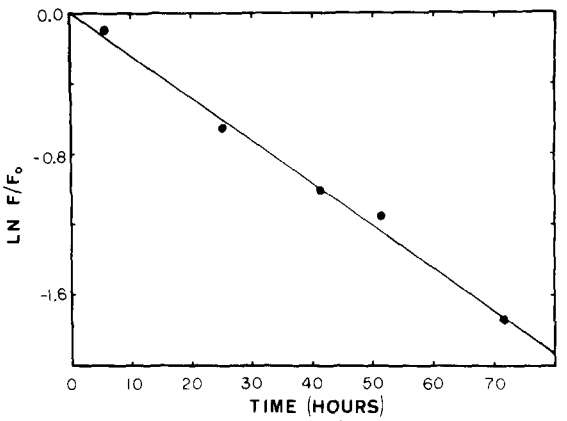

Fig. 3. Natural logarithm of the normalized DPH fluorescence intensity versus time in sonicated vesicles of previously oxidized egg phosphatidylcholine. The line results from a least-squares linear regression analysis of the data.

There are several possible mechanisms by which the fluorescence intensity of the DPH might be reduced in the presence of oxidizing lipids. Possible mechanisms include: (1) formation of an unexcitable ground-state complex between DPH and the lipid oxidation product; (2) excited-state quenching by a lipid oxidation product; (3) oxidized-lipid catalyzed, free-radical oxidation of DPH; and (4) formation of a covalent derivative of DPH with a lipid oxidation product. The first two mechanisms would be expected to produce kinetics identical to the kinetics of lipid oxidation. This was not observed. In order to rule out a simple quenching mechanism, the following experiment was performed. Sonicated, oxidized egg phosphatidylcholine vesicles containing non-fluorescing DPH were mixed with small, unilamellar vesicles prepared with non-oxidized egg phosphatidylcholine. In this mixture, the DPH should rapidly equilibrate between the two vesicle populations [4]

TABLE I

EFFECT OF LIPID OXIDATION ON DPH-DERIVED 'MICROVISCOSITY' PARAMETER

Lipid composition	Oxidation index	Fluorescence anisotropy	Fluorescence lifetime (nsec)	Lifetime-derived micro-viscosity (cpoise)	Intensity-derived micro-viscosity (cpoise)
Egg phosphatidylcholine	0.500	0.0982 ± 0.0011	7.6 ± 0.4	57.5	57.5
	0.979	0.0991 ± 0.0011	7.6 ± 0.6	58.5	18.7
25 mol% cholesterol/egg phosphatidylcholine	0.391	0.1554 ± 0.0018	8.2 ± 0.6	129.1	129.1
	0.914	0.1531 ± 0.0095	8.2 ± 0.5	125.6	15.1

(oxidized and non-oxidized). If associational quenching were the mechanism by which DPH fluorescence had been abolished, then any DPH molecules which transferred to a non-oxidized lipid environment should regain their fluorescence. The result of this experiment, however, was that no fluorescence recovery occurred. Furthermore, in the conjugate experiment (addition of non-oxidized egg phosphatidylcholine small, unilamellar vesicles containing fluorescing DPH to sonicated, oxidized egg phosphatidylcholine vesicles), there was no evidence of fluorescence quenching upon mixing of the two vesicle populations.

Additional insight into the mechanism of DPH fluorescence intensity loss is gained by noting that the lifetime of the DPH fluorescence excited state remained constant in oxidizing lipid vesicles even though the fluorescence intensity changed dramatically (Table I). This indicates that the fluorescence intensity reduction was not due to excited-state quenching. These results lead us to conclude that the loss of DPH fluorescence intensity associated with lipid oxidation is due either to oxidation of the DPH (mechanism 3) or the formation of a covalent complex between DPH and a lipid oxidation product (mechanism 4).

One of our motivations for undertaking this study was to establish the extent to which lipid oxidation could affect estimates of membrane fluidity obtained by using DPH. Table I illustrates the dramatic errors that can be introduced into the DPH-fluorescence-derived 'microviscosity' parameter when relative fluorescent intensities are used to approximate the fluorescence lifetime of DPH in oxidizable lipid membranes. The 'microviscosity' parameter, η, may be calculated [11] from measured anisotropy (R) and lifetime (τ) values as:

$$\eta(\tau) = \frac{C(R)\,T\tau}{(R_0/R) - 1}$$

or alternatively from DPH fluorescence intensity (F) measurements as:

$$\eta(F) = \frac{C(R)\,T(F/F_0)\,\tau_0}{(R_0/R) - 1}$$

[11]. The results in Table I illustrate that comparison of 'microviscosity' values derived from two oxidizable membrane preparations would be clearly invalid without direct measurement of DPH lifetimes in the two environments. However, in egg phosphatidylcholine (or egg phosphatidylcholine/cholesterol) multilamellar vesicles, the fluorescence anisotropy parameter (indicative of the rate and extent of hydrocarbon chain motion in the hydrophobic region of the bilayer [13]) was unaffected by lipid oxidation. Therefore, direct comparison of the DPH fluorescence anisotropy between two oxidizable environments would seem to be a more valid approach in the absence of lifetime measurements.

Acknowledgements

This work was supported by NSF Grant PCM76-16761, and carried out during the tenure of an Established Investigator Award to BRL from the American

Heart Association and with funds contributed in part by the North Carolina Heart Association.

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