A kinetic study of the oxidation effects of amphotericin B on human low-density lipoproteins

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Abstract The UV-visible results of this kinetic study show that amphothericin B as Fungizone is a much stronger oxidant than $CuSO_4$, itself a powerful oxidant of low-density lipoprotein (LDL). Amphotericin B as AmBisome alone has no oxidizing effect on LDL while a mixture of both AmBisome and $CuSO_4$ induces an important potentialization of the LDL oxidation. These results allow us to believe that the high toxicity of amphotericin B is related to its capacity to modify and to weaken the structure of LDL. In addition, differential scanning calorimetry experiments show that the oxidative modifications of LDL by $CuSO_4$ or by amphotericin B proceed through different mechanisms.

Key words: Oxidation; Low-density lipoprotein; Amphotericin B; Fungizone; AmBisome

1. Introduction

Amphotericin B (AmB), usually administered as Fungizone, a preparation using sodium deoxycholate, is the most powerful and effective antifungal drug used in the treatment of mycotic infections, but with a number of adverse effects, including myocardial toxicity [1].

Recent studies from our laboratory have established that the AmB species responsible, at least in part, for the in vivo toxicity, is a complex of the antibiotic with the low-density (LDL) and very-low-density lipoproteins. We have shown that hindering of this complex formation results in a decrease of the AmB toxicity [2], results confirmed by others [3]. In fact, Barwicz et al. [4] have shown that Fungizone strongly modified the LDL structure and enhanced the formation of lipid oxidation products. On the contrary, AmBisome, a less toxic liposomal formulation of AmB did not affect LDL oxidation [4]. If one takes into account that products of LDL oxidation are highly toxic [5–7] one can hypothesize that if AmB enhances this process, the AmB-lipoprotein interaction may, at least in part, be directly responsible for some of the toxic side-effects of the drug.

In this context, in order to get information on the induction of oxidation products of LDL by AmB, we have compared the kinetics of copper-induced oxidation of LDL with that induced by Fungizone or AmBisome, one of the techniques used being UV-vis spectroscopy. Our results show that Fungizone is a much stronger oxidant than $CuSO_4$, a powerful oxidant of lipoproteins. AmBisome alone has no oxidizing effect on LDL while a mixture of both AmBisome and $CuSO_4$ shows an important potentialization of the oxidation of the lipid part of LDL. On the other hand, the differential scanning calorimetry (DSC) thermograms are consistent with our findings that the oxidative modification of LDL by $CuSO_4$ or by AmB proceed through different mechanisms.

2. Materials and methods

2.1. Chemicals

Amphotericin B as Fungizone was obtained from Squibb, Canada. AmBisome was a generous gift of Vestar, CA, USA. The others chemicals were from usual sources and were used without further purification.

2.2. Lipoprotein preparation

Lipoprotein fractions were separated from fresh human plasma obtained from two healthy volunteers, by sequential preparative ultracentrifugation at 18°C, as described by Hatch and Lees [8]. The density-range-delivered LDL was 1.006–1.063 g/ml. The isolated LDL were dialyzed against 1.5 mM phosphate-buffered saline (PBS) containing no EDTA and sterilized with a porous filter (0.22 μm). The LDL solution obtained in this step was used as a stock solution and was stored at 7°C for no more than 20 days. The concentration of LDL was determined by the Lowry-Markwel method using bovine serum albumin as a standard. Cholesterol level was measured by enzymatic method and remained stable during 20 days. In all experiments the concentration of lipoprotein was 200 μg of protein/ml.

2.3. Preparation of the solutions

All the solutions (PBS, $CuSO_4$, Fungizone and AmBisome) were prepared in demineralized and double distilled water. The stock solution of AmB (0.26 mM) as Fungizone or AmBisome was always prepared immediately before use. The stock solution of $CuSO_4$ was 0.1 mM. For the UV-vis studies the final concentrations of AmB, $CuSO_4$ and LDL were 26 μM, 10 μM and 200 μg of protein/ml, respectively, using 1.5 mM PBS to obtain the desired volume. In order to record a sufficient signal, the concentrations used in the DSC experiments were 20 times larger.

2.4. UV-vis spectroscopy

The absorbance values and the absorption spectra were recorded using a Milton Roy Spectronic 3000 array spectrophotometer. For the kinetic experiments (which were run at 37°C during 24 h) the reference and sample cells were placed in a thermo-electric cuvette holder with a temperature stability of ±0.05°C, a probe directly monitored the temperature of the sample solution with ±0.3°C accuracy. The absorbance was recorded at 234 nm and at 680 nm every 3 min for the first 3 h and at longer intervals of time after that. Complete spectra were recorded a few times during the kinetic experiment.

2.5. Differential scanning calorimetry

The thermograms were recorded on a Hart Scientific Differential Scanning Calorimeter (Calorimetry Sciences, Provo, UT, USA) from 10 to 60°C at a scan rate of 10°C/h. The thermogram of the buffer used is subtracted from the thermogram obtained and then corrected for the thermal delay of the calorimeter.

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All the experimental results were repeated from three to seven times and were reproducible. The figures show the results of a representative experiment.

3. Results and discussion

It has been shown previously that a direct relationship was found between the interaction of AmB with LDL or VLDL and the in vivo toxicity of this drug [2,3]. It was demonstrated that LDL had a strong affinity to the aggregated form of AmB and that this interaction caused an oxidative modification of the lipoprotein. This oxidative reaction might be attributed to the well known property of AmB to form radicals of great chemical reactivity [9]. UV-vis spectroscopy has been used previously in the literature to determine the susceptibility of LDL to in vitro oxidation, particularly by monitoring the increase of absorbance at 234 nm [10–12]. At this wavelength the conjugated dienes resulting from phospholipid oxidation [10], as well as the oxidized cholesterol [11] absorb. In many recent studies a solution of $CuSO_4$ was used as a model system to monitor the physiological oxidation of the lipoprotein (e.g. [13,14]).

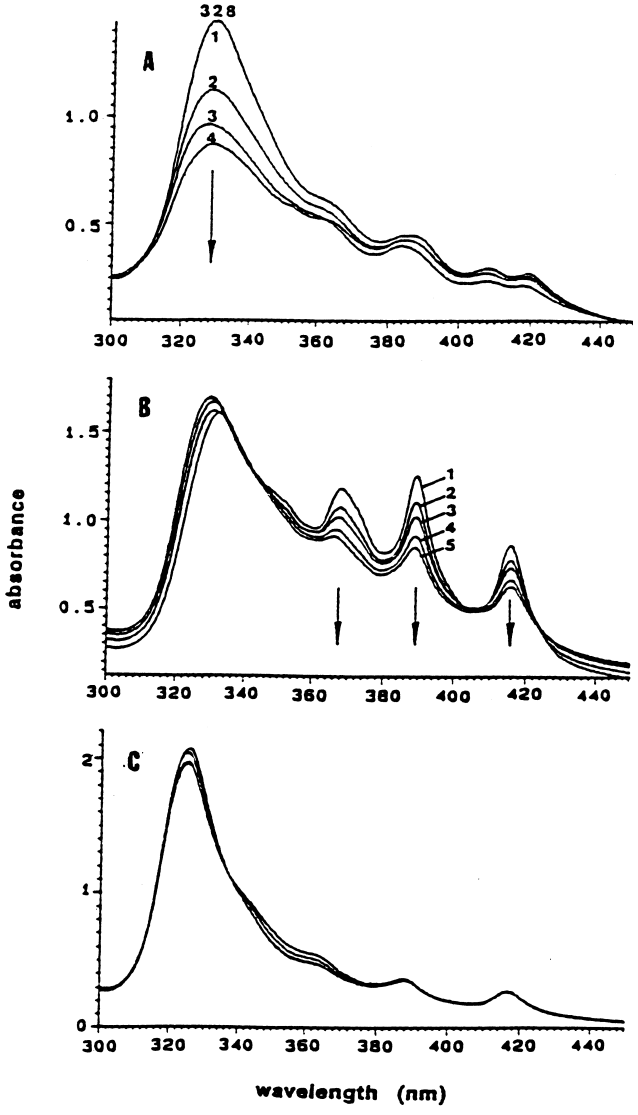

Fig. 1. Spectra of AmB, as Fungizone or AmBisome, in studied systems. A: Spectra of AmB (as Fungizone) alone or in mixture with $CuSO_4$. The arrow indicates a decrease of the absorption at 328 nm during a 24 h incubation at 37°C (1) 10 min; (2) 175 min; (3) 345 min; (4) 1440 min, i.e. 24 h, of incubation. B: Spectra of AmB as Fungizone in the presence of LDL. Arrows indicate the spectral changes during 24 h incubation at 37°C (1) 10 min; (2) 43 min; (3) 115 min; (4) 500 min; (5) 1440 min, i.e. 24 h of incubation. C: Spectra of AmB (as AmBisome) alone or in the presence of $CuSO_4$, LDL or $CuSO_4$+LDL, monitored during 24 h incubation at 37°C.

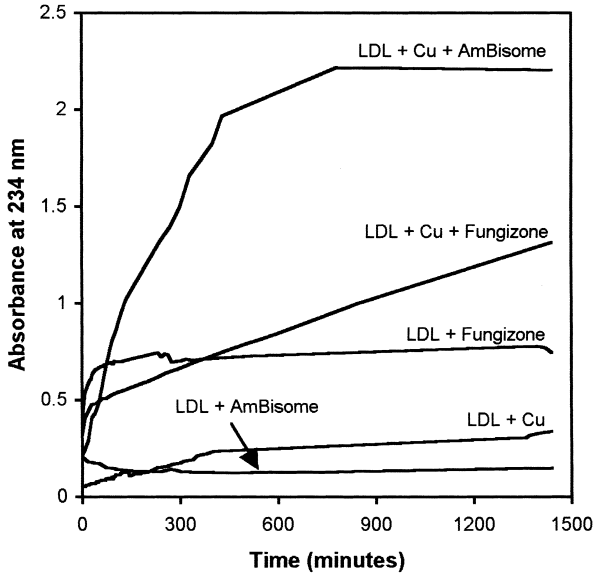

Fig. 2. Changes of the absorbance at 234 nm as a function of time to study the kinetics of the oxidative effect on LDL as a function of the various additives.

In the present study, we have compared the kinetics of copper-induced LDL oxidation with the oxidation induced by Fungizone or AmBisome alone. In addition, we have also studied the effect of a mixture of AmB (as Fungizone or AmBisome) together with $CuSO_4$ on the formation of LDL oxidation products. The changes in the UV-vis spectra of AmB during a 24 h incubation time at 37°C, were also monitored. Some of these spectra are presented in Fig. 1. In this figure the spectra of AmB (as Fungizone or as AmBisome) alone or in the presence of LDL, $CuSO_4$ or LDL and $CuSO_4$, are shown. Fig. 1A presents the spectra of AmB alone (as Fungizone) or in mixture with $CuSO_4$. The two series of spectra are identical, thereby showing that $CuSO_4$ does not affect the spectrum of the drug. The results showed, however, that a decrease in the absorption at 328 nm occurred during the incubation time. This indicates that a degradation of AmB, probably due to autoxidation, occurred, a well known phenomenon for AmB [9]. On the other hand, the spectra did not show modifications in the light scattering pattern (the absorbances at 300 nm or at 450 nm remained the same over 24 h), indicating that clusters of AmB are not formed as a function of time. In the presence of LDL the spectrum of AmB (as Fungizone) is different (Fig. 1B) and present the typical characteristics of a spectrum of AmB in interaction with cholesterol [2]. The spectral changes observed during the 24 h incubation indicate a decrease of this interaction (Fig. 1B, arrows) and a small increase in the light scattering

pattern. It is interesting to note that these changes started immediately after the addition of Fungizone to the LDL solution (compare spectrum 1 of Fig. 1A and B). Strikingly different results are observed when AmBisome is used instead of Fungizone. In this case, Fig. 1C shows that the spectra of AmB (as AmBisome) either alone, or in the presence of $CuSO_4$, or in the presence of LDL, or both $CuSO_4$ and LDL are identical and do not change during the 24 h incubation time at 37°C.

A comparison of the kinetics results obtained for all the systems under study is presented in Fig. 2 which shows the absorbance recorded at 234 nm plotted as a function of time. The results presented in this figure illustrate the net oxidative effect of the various additives on LDL because the solution of the lipoprotein alone, incubated under the same conditions as the samples under study, was placed in the reference cell. Therefore, the absorbance at 234 nm was automatically corrected for the natural autoxidation of LDL. In addition, Fungizone or AmBisome alone present a small absorption at this wavelength. However, it is important to note that this absorption is not changing either during the 24 h incubation time or when $CuSO_4$ is present in solution. The absorbance at 234 nm was thus also corrected for that absorption. In this context, it is observed that under the same conditions (24 h incubation at 37°C), AmB as Fungizone is a much stronger oxidant of LDL than $CuSO_4$. On the other hand, AmB as AmBisome seems to protect the lipoproteins against an oxidative process, especially during the first 4 h. In addition, since in the human body some oxidants may be present, it was interesting to study also the oxidative action of a mixture of Fungizone and $CuSO_4$ on LDL. In this case, the curve increases progressively during 24 h reaching an absorbance level at 234 nm twice that of the Fungizone+LDL sample. The situation is completely different when LDL is put in the presence of both AmBisome and $CuSO_4$. Here, a striking effect is observed: the oxidation of LDL caused by liposomal AmB (AmBisome) with $CuSO_4$ is more than seven times higher than the one caused by $CuSO_4$ alone. One may think that the important increase of the signal observed with AmBisome in the presence of $CuSO_4$ might be due to an aggregation of the small vesicles of AmBisome, thus leading to an increase of the light scattering. However, this is not the case since the absorbance at 234 nm does not change at all when $CuSO_4$ is put in presence of AmBisome alone during the whole incubation time.

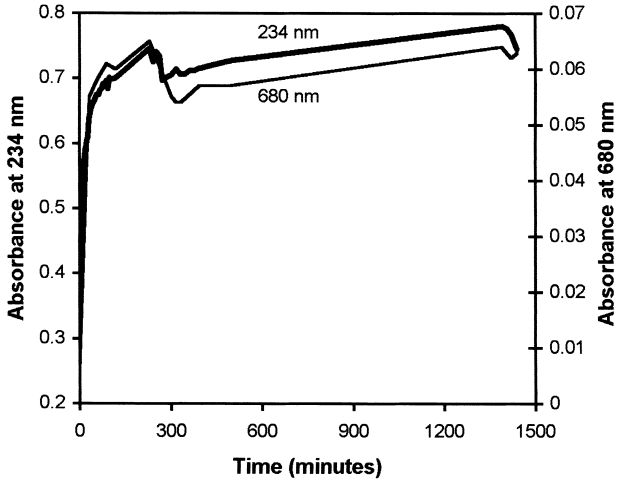

Fig. 3. The kinetics of LDL oxidation induced by AmB (as Fungizone) as monitored by the changes in the absorbance at 234 nm (in bold), together with the variation of the light scattering pattern (absorbance at 680 nm) as a function of time.

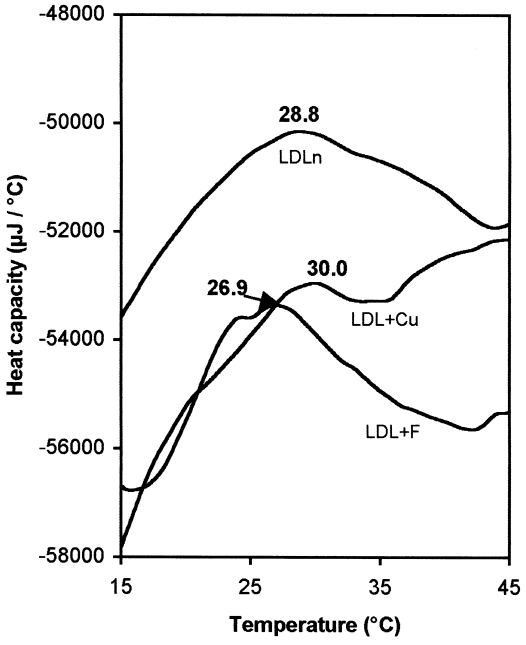

Fig. 4. The thermograms of native LDL (LDLn), and LDL mixed with $CuSO_4$ (LDL+Cu) or Fungizone (LDL+F). The transition temperatures are indicated on each thermogram.

It is thus very surprising that despite the fact that AmBisome alone has no oxidizing effect on LDL and despite the fact that $CuSO_4$ has also by itself no effect on AmBisome (Fig. 1C), a mixture of both AmBisome and $CuSO_4$ shows such an important potentialization of the LDL oxidation. It may thus be suggested that the low-toxic formulation of AmB (AmBisome), might become a powerful oxidant if oxidative conditions, such as in atherosclerotic lesions for example, are already present.

Our results therefore show that a delicate redox equilibrium is created under the present experimental conditions, owing to the characteristics of the components used here. The main elements of this equilibrium are: the facility of AmB for autoxidation, the great susceptibility of LDL to oxidation, mainly in the presence of free radicals, and the oxidative effect of Cu^{2+} ions. Lately, it was demonstrated that AmB contained in Fungizone extracted cholesterol from LDL, thus sensitizing them to oxidation [4]. This effect would probably represent the basic step to create a new red–ox equilibrium. AmBisome, on the other hand, is not sensitive to oxidation, does not bind to the LDL cholesterol and thus does not contribute to the oxidation of the lipoprotein.

Aggregation represents one of the main characteristics of strongly oxidized LDL [6,15,16]. Thus Fig. 3 presents the kinetics of the oxidation of LDL induced by Fungizone (as taken from Fig. 2) together with the variation of the light scattering with time, as monitored by the absorbance at 680 nm. The great similarity between these two curves strongly suggest that the aggregation of LDL is closely related to its oxidation.

In order to further investigate the effect of Fungizone on the lipid component of LDL, Fig. 4 illustrates the thermograms recorded for native LDL, LDL in mixture with Fungizone or with $CuSO_4$. It shows the typical broad transitions usually observed for LDL [17–19]. The results indicate that the transition centered at 28.8°C for native LDL is decreased to 26.9°C when Fungizone is used. On the other hand, it increases to 30.0°C when $CuSO_4$ is used to oxidize LDL, in good correlation with the literature data [18]. It is thus clear that $CuSO_4$ and Fungizone have different effects: while $CuSO_4$ contributes to a certain rigidification of the lipid components that are involved in the thermotropic transition of LDL, to the contrary, Fungizone seems to fluidify these lipid components. Thus, $CuSO_4$ and Fungizone act differently towards the lipid part of LDL, thereby showing that the structure of LDL is modified by two different mechanisms.

In conclusion, therefore, the results presented here have shown that AmB as Fungizone, but not as AmBisome, is a powerful oxidant of LDL, even more powerful than $CuSO_4$, itself considered as a strong oxidant of LDL. However, under oxidizing conditions, a potentialization of the oxidation power is observed, this effect being much more important for AmBisome than for Fungizone. It is known that treatment with AmB has severe, non-reversible, side effects, such as nephrotoxicity and cardiac complications [20,21]. Lately, a new study [22] has demonstrated that AmB infusion in rats may induce the formation of free radicals (lipid peroxides) in various organs such as brain, lungs, and kidneys, increasing the lipid peroxide level two, three, and almost four times, respectively. In this context, the fact that AmB presents a great affinity to the lipid part of LDL, thus contributing to the oxidation of its lipoprotein fraction, has important consequences that have to be taken into account in the treatment of fungal infections, particularly in the case of patients with cardiovascular or kidney weaknesses.

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