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Original Contribution

DUAL EFFECT OF GLUCOSE ON LDL OXIDATION: DEPENDENCE ON VITAMIN E

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Abstract—The aim of the present study was to determine the direct effect of glucose on LDL oxidation, a key step in the development of atherosclerosis. Purified human LDL were incubated with glucose (500 mg/dl) and LDL oxidation was started by adding $CuCl_2$ to the media. Glucose delayed the vitamin E consumption, but accelerated the formation of conjugated dienes and increased both the formation of thiobarbituric acid reacting substances (TBARS) and LDL electrophoretic mobility. When LDL were incubated with increasing concentrations of glucose and submitted to oxidation, the formation of conjugated dienes, TBARS, and the electrophoretic mobility increased in a concentration-dependent manner. When LDL was enriched with vitamin E, it showed a delay in the formation of conjugated dienes, even in the presence of glucose. To determine whether glucose had any effect on LDL oxidation, once the process was started and vitamin E consumed, LDL were submitted to oxidation and, at different times thereafter, glucose was added into the media. Under these conditions glucose also accelerated the LDL oxidation. In summary, present results show that in LDL submitted to oxidation, glucose delays the early phases of the oxidation, slowing the vitamin E consumption, but it accelerates the rate of LDL oxidation once LDL vitamin E has been consumed; the effect being concentration-dependent. © 2002 Elsevier Science Inc.

Keywords—Low density lipoprotein, Glucose, Vitamin E, Free radicals

INTRODUCTION

Among subjects with insulin-dependent diabetes mellitus (IDDM), cardiovascular diseases secondary to atherosclerosis are the leading cause of death [1,2]. Numerous studies have been conducted to determine the risk factors involved in this accelerated process, with inconclusive results, although there is clear evidence that high glucose concentrations are involved in most of the complications secondary to IDDM [3–5], including the atherosclerotic process.

LDL oxidation and free radical generation seem to play a central role in the atherosclerotic process [6–9]. There are several reports showing in poorly controlled diabetic subjects increased levels of lipid peroxides [10], increased LDL susceptibility to oxidation [11,12], and decreased levels of antioxidants [11,13,14], linking the oxidation of LDL to their accelerated atherosclerotic process. In fact, in isolated LDL preparations and in the cell culture model, it has been shown that LDL oxidation is accelerated by high glucose concentrations [15,16].

The mechanisms involved in the association between glucose, LDL oxidation, and atherosclerosis are not well understood. The formation in the LDL of Amadori products secondary to protein glycosylation makes these particles more atherogenic [17,18], although this process seems unlikely to play a mayor role in vivo, as it takes weeks for these products to be generated [19], and the LDL half life is only a few days [20]. In a red cell model of oxidative damage, it has been shown that glucose may act either as an antioxidant or pro-oxidant, depending on the cell concentration of vitamin E [21], in a manner similar to what we and others have shown for vitamin C, dehydroascorbic acid, and flavonoids [22–25]. These compounds may act either as LDL antioxidant or pro-oxidant, depending on the LDL vitamin E content. When vitamin E is present in the LDL those compounds act as antioxidant, whereas when added during the process of

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the LDL oxidation, once vitamin E has been consumed [22] or to LDL minimally oxidized [23,24], they act as pro-oxidant, accelerating the oxidation of the LDL lipids. Because glucose has a structure similar to ascorbic acid, it is hypothesized that the effects of glucose may be antioxidant or pro-oxidant, depending on the degree of the LDL oxidation and/or the LDL vitamin E content.

Thus, the aim of the present study was to determine whether concentrations of glucose, frequently found in IDDM subjects, have a different effect on the LDL oxidation depending on their vitamin E content.

MATERIALS AND METHODS

Isolation of LDL

LDL were isolated from ethylenediaminetetraacetic acid (EDTA)-treated plasma obtained from different healthy donors, after 12 h fasting, by ultracentrifugation and stored in a sucrose solution [26] as described previously [22,27].

Because most of the chemicals commonly used in the laboratory, including glucose and phosphate-buffered saline, may contain metal ions as contaminants, all the solutions used in the experiments were treated with Chelex (chelating resing iminodiacetic acid; Sigma, Barcelona, Spain) to remove any metal traces.

LDL vitamin E enrichment was performed using the method described by Esterbauer et al. [28]. In brief, EDTA-treated plasma (10 ml) was incubated with 100 μl of a solution of α-tocopherol 100 mM previously diluted in ethanol for 4 h in a shaking bath at 37°C, under sterile conditions. In parallel, the same volume of plasma was incubated with 100 μl of ethanol. LDL were isolated and stored as described above.

Determination of the effects of glucose on the LDL oxidation

After being thawed, the LDL were passed through a Sephacryl-400 column (Pharmacia LKB, Biotechnology, Inc., Madrid, Spain) to remove EDTA and sucrose. LDL protein concentration was determined immediately using the Lowry procedure [29] and aliquots of 0.1 mg of LDL protein/ml were incubated with glucose (500 mg/dl) and $CuCl_2$ (2.4 μM). Butylated hydroxytoluene (BHT) and EDTA (final concentration 1 mM) were added to aliquots obtained at different times to stop the ongoing oxidation process. These aliquots were used to test different parameters related to the degree of LDL oxidation, including: (i) vitamin E concentration, which was determined by high-performance liquid chromatography following the method described previously [22,27]; (ii) mobility on agarose gel electrophoresis; (iii) the amount of thiobarbituric acid reacting substances (TBARS), which was measured by using malondialdehyde (MDA) formed from 1,1,3,3-tetramethoxypropane as standard [30]; and (iv) conjugated dienes formation, which was determined as described by Esterbauer et al. [31]. In short, 1 ml of the LDL solution was incubated in a quartz cuvette at 37°C. Absorbance was read at 234 nm, in a Beckman DU-640 spectrophotometer, every 10 min for a maximum of 8 h or until the rapid phase of LDL oxidation reached a plateau. The lag phase and the slope were determined. The lag phase was estimated as the incubation time corresponding to the intersection of two lines drawn from the changes in optical density, one through the initial, slowly rising curve that corresponds to the utilization of the endogenous antioxidants and the other, a subsequent, rapidly rising curve that corresponds to the propagation phase of the LDL oxidation, when most of the fatty acids are being oxidized [31]. The lag phase is expressed as minutes from the addition of $CuCl_2$. The slope was determined by a linear regression.

The effects of glucose on the formation of conjugated dienes was followed in LDL obtained from plasma treated with vitamin E and compared with LDL obtained from the same plasma without the addition of vitamin E.

To study whether the effects of glucose were concen-

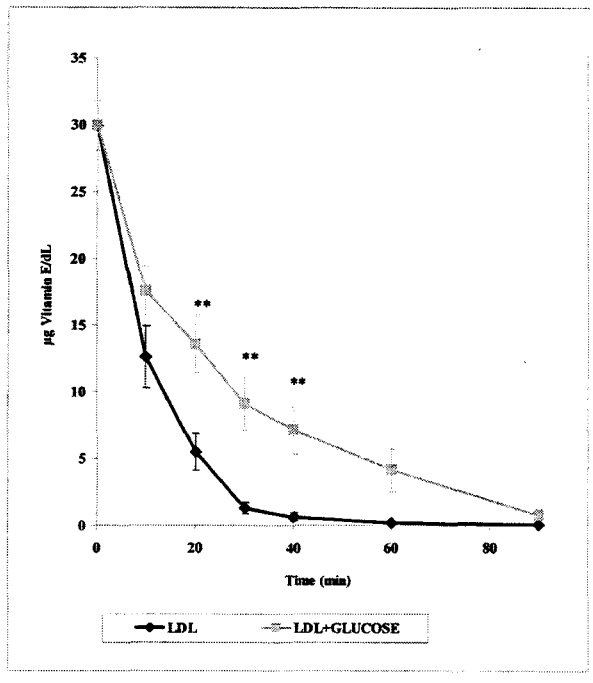


Fig. 1. Effects of glucose on the consumption of vitamin E in LDL. LDL (0.1 mg of LDL protein/ml) was incubated in a media containing glucose (500 mg/dl). Oxidation was induced with the addition of $CuCl_2$ (2.4 μM). At different times, aliquots were obtained and vitamin E determined, as described in the Materials and Methods section. Results are expressed as the mean ± SEM of six different samples. *LDL incubated with vs. without glucose. *$p < .05$; **$p < .01$.

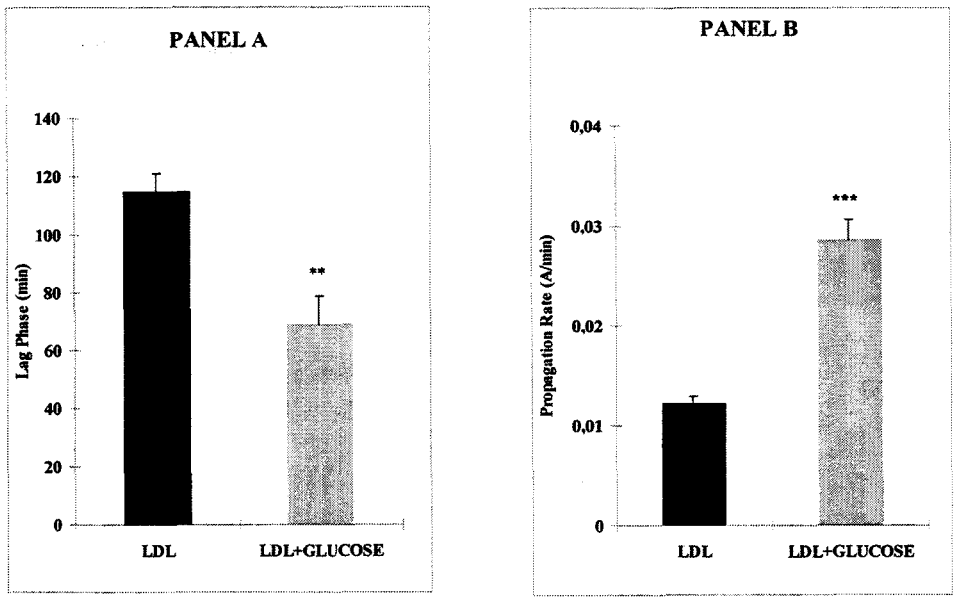


Fig. 2. Effects of glucose on the formation of conjugated dienes in LDL incubated in vitro and submitted to $CuCl_2$ oxidation as described in Fig. 1. (A) Lag phase; (B) propagation rate. Results are expressed as the mean ± SEM of six different samples. *LDL incubated with vs. without glucose. $**p < .01$; $***p < .001$.

tration-dependent, the LDL solution was incubated with different concentrations of glucose, ranging from 0 to 500 mg/dl, immediately before $CuCl_2$ addition. Conjugated dienes formation, electrophoretic mobility, and TBARS production were determined as above.

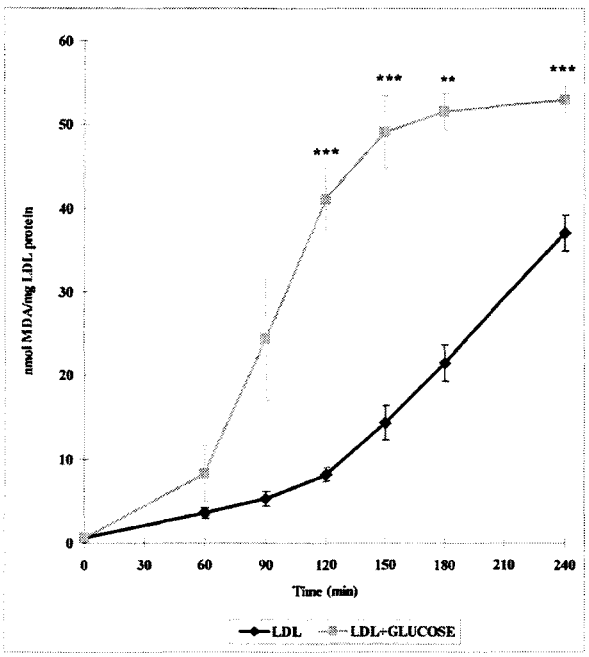


Fig. 3. Effects of glucose on TBARS formation by $CuCl_2$ mediated LDL oxidation. The experiment design is described in Fig. 1. Results are expressed as the mean ± SEM of six different samples. *LDL incubated with vs. without glucose. $**p < .01$; $***p < .001$.

In another set of experiments, glucose (500 mg/dl) was added at different times (20, 40, or 70 min) after the initiation of the LDL oxidation process.

To determine whether glucose per se could initiate the LDL oxidation process, LDL was incubated with glucose (500 mg/dl) in the absence of $CuCl_2$ to let the oxidation process develop spontaneously. In these two experiments, the formation of conjugated dienes and the LDL electrophoretic mobility were also determined.

Statistical analysis

Results are expressed as the mean ± SEM. The significance of the difference between the means of samples incubated under different conditions were analyzed statistically using analysis of variance and the Tukey test. The Systat program was used for the statistical analysis (Systat, Inc. Evanston, IL, USA).

RESULTS

Because the first products to be consumed during LDL oxidation process are the endogenous antioxidants, mainly vitamin E, in order to determine the effects of glucose upon this process, LDL were submitted to oxidation with $CuCl_2$ in a media containing glucose (500 mg/dl). As shown in Fig. 1, the presence of glucose in the media delayed the consumption of vitamin E, when compared to the same LDL solution in a media without glucose. However, as shown in Fig. 2, when other pa-

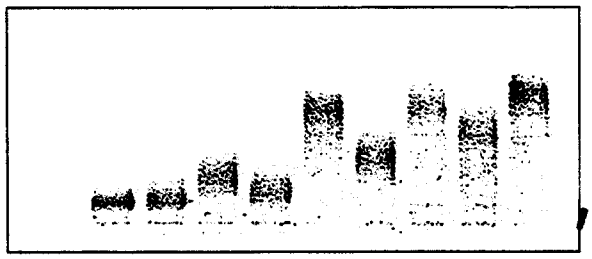


Fig. 4. Effects of glucose on LDL electrophoretic mobility. The experiment design is described in Fig. 1. The electrophoretic mobility was determined at different times (60, 120, 180, and 240 min) after the addition of $CuCl_2$. This representative experiment was repeated with LDL obtained from six different LDL samples with similar results.

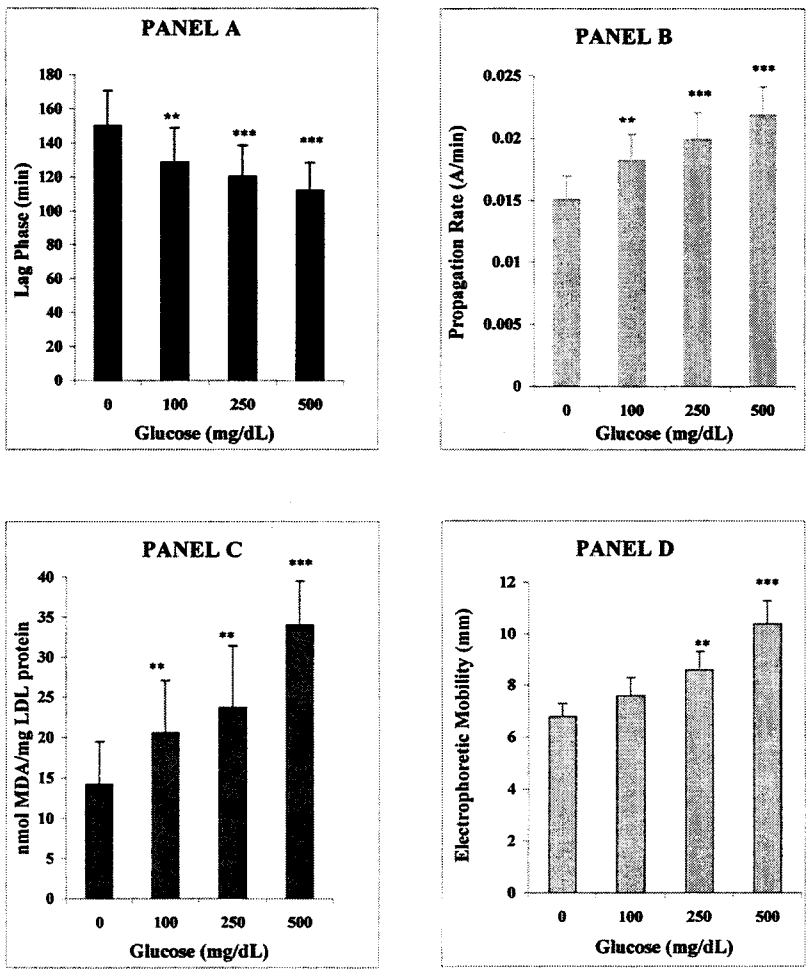


Fig. 5. Effects of different concentrations of glucose on LDL submitted to oxidation as described in Fig. 1. (A) and (B) shows, respectively, the lag phase and the propagation rate of the formation of conjugated dienes; and (C) and (D) show, respectively, the TBARS and the electrophoretic mobility of the LDL particles submitted to oxidation for 150 min. The LDL oxidation was stopped as described in the Materials and Methods section by adding BHT and EDTA (final concentration 1 mM). Results are expressed as the mean ± SEM of eight different samples. *LDL incubated with vs. without glucose. $^{**}p < .01$; $^{***}p < .001$.

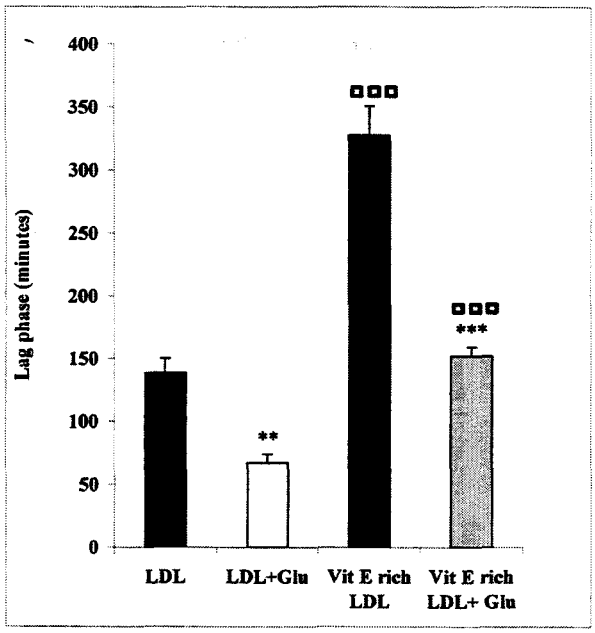


Fig. 6. Effects of glucose on the formation of conjugated dienes in vitamin E-enriched LDL. LDL were incubated in vitro and submitted to $CuCl_2$ oxidation as described in Fig. 1. The mean LDL vitamin E content in the LDL obtained from plasma treated with vitamin E, as described in the Materials and Methods section was 94 ± 5.84 (μg of vitamin E/dl) vs. 23.07 ± 2.07 (μg of vitamin E/dl) in the LDL from nontreated plasma. Results are expressed as the mean ± SEM of six different samples. *LDL incubated with vs. without glucose. $^{**}p < .01$; $^{***}p < .001$. □ LDL enriched with vitamin E vs. nonenriched LDL. □□□ $p < .001$.

rameters related to more advanced phases of the LDL oxidation process were tested, the incubation of LDL with glucose decreased the lag phase in the formation of conjugated dienes and increased the propagation rate. The formation of TBARS and the LDL electrophoretic mobility, variables related to lipid oxidation and LDL protein modification, respectively, also increased in LDL submitted to oxidation in a media containing glucose (Figs. 3 and 4).

To study whether the glucose effects were concentration-dependent, LDL were submitted to oxidation with $CuCl_2$ in a media containing increasing concentrations of glucose (0, 100, 250, and 500 mg/dl). As shown in Fig. 5, the lag phase decreased (Fig. 5A) and the propagation rate (Fig. 5B) of the formation of conjugated dienes, the TBARS concentration (Fig. 5C), and the electrophoretic mobility (Fig. 5D) increased in a glucose concentration-dependent manner.

When vitamin E-enriched LDL were submitted to oxidation with $CuCl_2$, as expected, a marked delay in the formation of conjugated dienes was observed (Fig. 6). Under these conditions, the addition of glucose (500 mg/dl) to the media accelerated the formation of conjugated dienes, although the lag phase in the vitamin E-enriched LDL was longer than in the nonenriched LDL (Fig. 6).

To study whether glucose could accelerate the oxidation once the process was started, in another set of experiments, glucose (500 mg/dl) was added to the media containing the oxidizing LDL at different times after the addition of $CuCl_2$. As shown in Figs. 7 and 8 the addition of glucose clearly accelerated the oxidation process, as the increase in the propagation rate of conjugated dienes formation (Fig. 7) and in the LDL electrophoretic mobility (Fig. 8) shows.

To determine whether glucose, per se, in the absence of metal ions could modify the oxidation of LDL, LDL were incubated with glucose (500 mg/dl) as above, but in the absence of $CuCl_2$ and let to spontaneous oxidation. Under these conditions, after 8 h no changes were observed in the absorbance at 234 nm, indicating that LDL was not oxidized (data not shown).

Finally, it was tested whether high glucose concentrations alone incubated with $CuCl_2$ (2.4 μM) could induce changes in the optical density (OD) at 234 nm, because it could interfere with the determination of conjugated dienes from the LDL. Glucose (500 mg/dl) incubated with $CuCl_2$ (2.4 μM) without LDL in the media did not change the absorbance at 234 nm (data not shown).

DISCUSSION

Present results show for the first time that the disappearance of vitamin E in LDL subjected to oxidation by transitional metal ions is delayed by a glucose concentration frequently found in subjects with poorly controlled IDDM. Because vitamin E is one of the first products to be consumed during the LDL oxidation process [32], this finding indicates that glucose delays the early phases of LDL oxidation. However, high glucose concentration accelerated the LDL oxidation once vitamin E was consumed, as shown here by the decreased lag phase and the increased slope in the formation of conjugated dienes, the enhanced formation of TBARS, and the higher electrophoretic mobility.

In the present model of LDL oxidation, we hypothesize that the free radicals generated in the water phase by the copper, will be taken up by the vitamin E of the LDL surface and by the glucose of the media, therefore, delaying the vitamin E consumption, as our results show. As the process continues and the LDL vitamin E is consumed, the oxidation of fatty acids starts generating peroxides, which, in a media containing copper, as has been described in other systems containing transitional metal ions, can also oxidize the glucose, generating glucose-derived aldehydes [33]. Both the oxidation of glucose and glucose-derived aldehydes will generate more

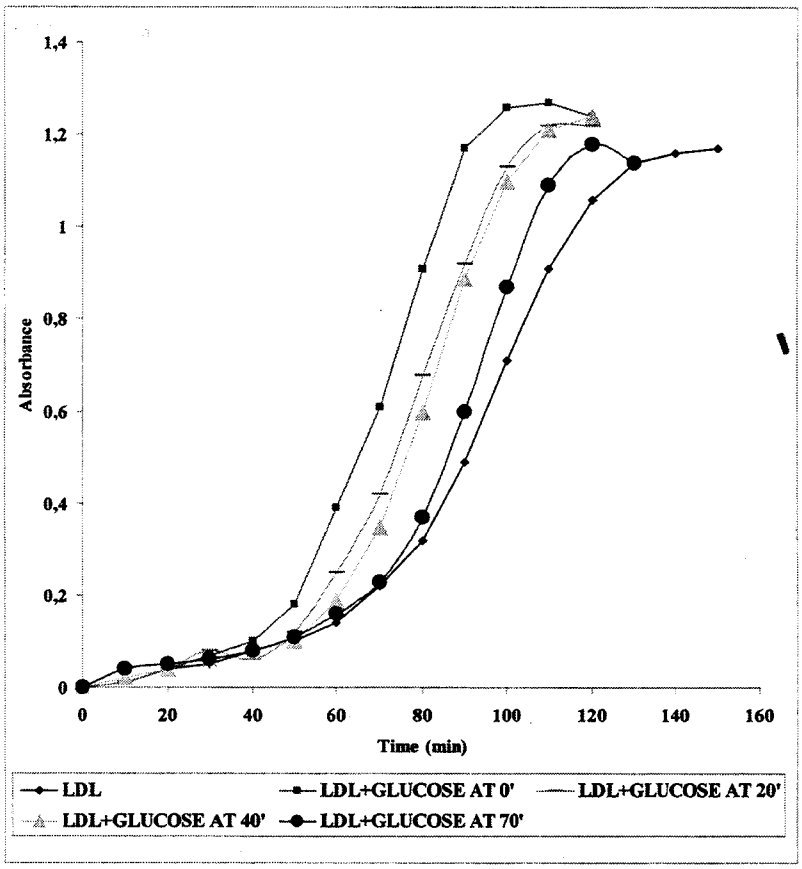
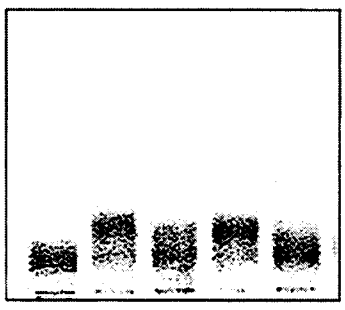


Fig. 7. Effects of glucose on LDL submitted to different degrees of oxidation. LDL was submitted to oxidation with the addition of $CuCl_2$ (2.4 μM) and the formation of conjugated dienes was followed as described in the Materials and Methods section. At different times (0, 20, 40, and 70 min) after the initiation of the oxidation process, glucose was added into the media to a final concentration of 500 mg/dl. The experiment was reproduced with LDL from four different donors with similar results.



Addition Time	0	20	40	70
Glucose Addition	-	+	+	+

Fig. 8. Electrophoretic mobility of LDL submitted to different degrees of oxidation incubated with glucose. The same procedure as described in Fig. 7 was followed in this experiment. The oxidation process was stopped at 90 min by adding BHT and EDTA (final concentration 1 mM). The same experiment was repeated with LDL from four different donors with similar results.

free radicals, accelerating the LDL fatty acids oxidation, as described by Hunt et al. [34]. This mechanism is very similar to the one proposed to explain the antioxidant and pro-oxidant effects of ascorbic acid [35]. It is worthwhile to remember the structural similarity between ascorbic acid and glucose and the fact that glucose and ascorbic acid when submitted to oxidation may generate the same aldehydes [33]. We cannot rule out that glucose, like ascorbic acid, could reduce the oxidized copper in the presence of lipid peroxides, as has been described [34–38]. A phenomenon like this could also lead to an acceleration of the LDL oxidation, by allowing further generation of free radicals from the copper oxidation [34, 35]. Therefore, glucose as other compounds like ascorbic acid, dehydroascorbic acid, or flavonoids [22] could act either as LDL antioxidant or pro-oxidant, depending on the presence of vitamin E in the LDL.

If a process like the one described occurred in vivo and in LDL oxidized under different conditions it might explain the accelerated atherosclerotic process observed

in subjects with IDDM. Even if, under basal conditions, LDL of the diabetic subjects were similar to the nondiabetic, when the oxidation process starts and the vitamin E is consumed, the presence of glucose in the media would accelerate the oxidation process, increasing the consequences of the oxidized LDL, including the accelerated atherosclerotic process. What it is even more relevant from present results is the fact that LDL with higher content of vitamin E will be less susceptible to the effects of glucose on LDL oxidation and, therefore, if a similar mechanism would occur in vivo, less prone to the development of atherosclerosis. Our data have demonstrated that increasing the vitamin E content of the LDL, the glucose pro-oxidant effects are markedly reduced. In contrast, the diabetic subjects with the lowest intake of vitamin E, and therefore lower levels of vitamin E in their LDL would be more susceptible to the effects of the hyperglycemia. It is remarkable that some studies in humans have shown lower levels of vitamin E in IDDM subjects [13,14], making them more susceptible to the pro-oxidant effects of the hyperglycemia. Also, several studies have shown in the experimental animal model that some of the complications secondary to diabetes can be prevented with the administration of antioxidants, including vitamin E [39–41], despite no improvement of the hyperglycemia. What is even more relevant from present results is the possibility of avoiding the effects of glucose on LDL oxidation by increasing the vitamin E content of these particles.

We have also shown here, that the pro-oxidant effect of glucose is concentration-dependent, which would be in agreement with the fact that IDDM subjects with complications secondary to diabetes are related to the degree of metabolic control, those with the highest levels of glucose have the highest risk of developing atherosclerosis and cardiovascular diseases [3,4].

As described recently by Halliwell [42], there is increasing evidence that, in the general population, not everybody will be subjected to the same oxidative stress, and some subjects, probably including patients with diabetes, may be at a higher risk of developing the complications of an increased oxidative stress. Present findings would indicate that this population may benefit from a higher intake of vitamin E than the current recommendations.

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ABBREVIATIONS

BHT—butylated hydroxytoluene
CVD—cardiovascular diseases
HPLC—high-performance liquid chromatography
IDDM—insulin-dependent diabetes mellitus
LDL—low density lipoprotein
TBARS—thiobarbituric acid reacting substances