Vol. 148, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1987 Pages 260-266

# TOXICITY OF ENZYMICALLY-OXIDIZED LOW-DENSITY LIPOPROTEIN

Alan W. Bernheimer[a], William G. Robinson[b], Regina Linder*, Deborra Mullins[c], Y. K. Yip[d], Norman S. Cooper[+], Irving Seidman[e], and T. Uwajima[§]

[a] Department of Microbiology, [b] Department of Biochemistry, [c] Department of Cell Biology, [d] Department of Microbiology and Kaplan Cancer Center, [e] Department of Pathology, New York University Medical Center, New York, NY 10016
* Hunter College School of Health Sciences, New York, NY 10010
+ Department of Pathology, New York University - VA Medical Centers, New York, NY 10016
§ Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd., Machidashi, Tokyo, Japan

Received August 26, 1987

Intravenous injection of cholesterol oxidase into hyperlipidemic rabbits in which aortic atheromatous lesions have been induced by dietary means is lethal within hours, whereas injection of the same enzyme into normal rabbits has no visible adverse effect. The lethal effect of the enzyme is explicable by the finding that injection of cholesterol-oxidase treated low-density lipoprotein kills normal rabbits, in contrast to untreated low-density lipoprotein which does not. Enzymically oxidized low-density lipoprotein was also found to be cytotoxic for two human cell lines and for cultured bovine aortic endothelial cells. We suggest that in vivo enzymic conversion of low-density lipoprotein cholesterol to low-density lipoprotein cholestenone may possibly play a role in the initiation of atheromatous lesions in humans.


Cholesterol oxidase (3β-hydroxy-steroid oxidase; EC 1.1.3.6) catalyzes the oxidation of cholesterol to $\Delta^4$-cholestenone (4-cholesten-3-one). In this reaction the β-hydroxyl group of cholesterol is oxidized to a ketone, and the double bond shifts from the $\Delta^5$ to the $\Delta^4$ position. The properties of this enzyme have been reviewed in detail (1). In a study of its effect on experimentally induced atheromatosis, we observed that rabbits fed a 1% cholesterol diet died following injection of the enzyme, whereas rabbits fed a normal diet survived (Table 1). Postmortem examination of the former revealed neither gross nor histological abnormalities, except for atheromatous plaques in the aortas of all cholesterol-fed rabbits, and some intravascular hemolysis in one of them. The enzyme also proved lethal for a

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein; LDL-one, cholestenone-containing low-density lipoprotein.

0006-291X/87 $1.50

TABLE 1. Lethal Effect of cholesterol oxidase in cholesterol-fed and other rabbits

| Strain | Prior treatment | Number of Rabbits | Cholesterol oxidase injected‡ | Result |
|---|---|---|---|---|
| New Zealand White | 20 weeks on high cholesterol diet+ | 3 | 150 units (Prep A) | All died in 24 hrs |
| New Zealand White | 11 weeks on high cholesterol diet+ | 6 | 3.2 units (Prep B) | 4 died in 24 hrs, 1 died in 32 to 48 hrs |
| New Zealand White | Normal diet | 3 | 3.2 units (Prep B) | All survived |
| Watanabe* | Normal diet | 1 | 3.2 units (Prep B) | Died in 3 hrs |

* Selectively bred strain having hypercholesteremia and significantly increased incidence of coronary atherosclerosis (2).

\+ Commercial diet (Lab Rabbit Chow, 5321, Ralson Purina Co., St. Louis, MO) containing one percent cholesterol.

‡ Prep A was cholesterol oxidase from Brevibacterium sp., purchased from Beckman Instruments, Inc., Palo Alto, CA. Its specific activity was 10 units per mg. Prep B was cholesterol oxidase from Brevibacterium sterolicum (5) provided by Kyowa Hakko Co., Ltd., Machidashi, Tokyo, Japan. It contained one part of enzyme and 99 parts of bovine serum albumin, the latter added as a stabilizing agent. The enzyme alone had a specific activity of 16 units per mg.

Watanabe rabbit (2). These results are not in agreement with those of Imshenetskii et al. (3) who mentioned no toxic effects following injection of hypercholesterolemic rabbits with cholesterol oxidase contained in a crude extract from the mycelium of Actinomyces lavendulae.

To find out whether the phenomenon seen in rabbits could be demonstrated in a smaller animal, 12 Swiss Webster albino mice weighing 18 to 20 g were maintained on 1% cholesterol feed of the same composition as that used for rabbits. Since the mice weighed about one one-hundredth as much as the rabbits, they were intravenously injected, in groups of three, with one one-hundredth the amount of cholesterol oxidase used for rabbits, after feeding on the cholesterol diet for 2, 4, 8 and 12 weeks. All survived without evidence of ill effect. We conclude that the phenomenon observed in rabbits cannot be reproduced in mice under the conditions used.

Cholesterol oxidase is a flavoprotein whose action on cholesterol generates hydrogen peroxide and 4-cholesten-3-one (hereafter designated cholestenone). In vivo, hydrogen peroxide is quickly converted to water and oxygen, and if a sufficient amount of the latter were to accumulate, gas embolism could conceivably account for death. It can be calculated, however,

TABLE 2. Toxicity of enzymically oxidized LDL for normal animals

| Animal species | Number of animals | Material injected* | Effect |
|---|---|---|---|
| Rabbit | 2 | cholesterol-oxidase treated LDL | Both died in 8 to 24 hrs.+ |
| Rabbit | 2 | untreated LDL | Both survived |
| Rabbit | 2 | cholesterol-oxidase treated HDL | One died in 8 to 24 hrs.+ One survived |
| Guinea pig | 2 | Cholesterol-oxidase treated LDL | Both survived |
| Mouse | 4 | Cholesterol-oxidase treated LDL | All survived |

* Human LDL and HDL were isolated by sequential differential ultracentrifugation of outdated, unfrozen human plasma as described by Havel et al. (6). The fractions were sterilized by filtration (Nalge, 0.45 μm) and stored at 4°C. In rabbit experiments, 3.2 units of cholesterol oxidase (Prep B, corresponding to 0.2 mg pure enzyme) were incubated for 2 hrs at 37°C with a volume of LDL or HDL containing a total of 51 mg cholesterol. The whole mixture was injected intracardially into each rabbit. In guinea pig experiments, an identical mixture of LDL and enzyme was used, but the amount injected intracardially was adjusted for difference in body weights. Prior to injection, guinea pigs were injected intraperitoneally with a tranquilizing dose of nembutal in order to facilitate intracardial injection. The same ratio of enzyme to LDL was used in mice, injected intravenously, but with 10 to 20 times as much mixture, on a weight basis.

\+ Showed nasal hemorrhage.

that the maximum amount of hydrogen peroxide that could be produced is very small and it is known, moreover, that animals can tolerate substantial quantities of intravenously administered hydrogen peroxide (4).

The foregoing information suggests that enzymic modification of LDL, a serum component having a high content of cholesterol, and generally regarded as important for the development of atherosclerosis, might account for the lethal effect. Experiments were done to test the idea that conversion of LDL to LDL whose unesterified cholesterol was enzymically oxidized in vitro to cholestenone, could result in a product, LDL-one, that would prove lethal for normal rabbits.

As shown by gas-liquid chromatography, in vitro incubation of a standard mixture of LDL and cholesterol oxidase results in disappearance of about 80% of the cholesterol, and appearance of a new peak which was identical with that obtained for cholestenone alone. Cholesterol esters were not modified, and there was no change in tocopherol content. The data of Table 2 show that enzymically oxidized LDL is indeed lethal for rabbits. In contrast, guinea

TABLE 3. Effect of cholesterol oxidase (CO) treated LDL and HDL on viability of Daudi and U937 cells as determined by [$^3$H]-thymidine incorporation*

| Test material | Cells | Counts per minute as percent of control | | |
|---|---|---|---|---|
| | | Trial 1 | Trial 2 | Trial 3 |
| None | Daudi | - | 100 | 100 |
| | U937 | 100 | 100 | 100 |
| CO-treated LDL | Daudi | - | 0.9 | 57 |
| | U937 | 8.5 | 0.3 | 0.6 |
| CO-treated HDL | Daudi | - | 37 | 86 |
| | U937 | - | 102 | 100 |
| LDL only | Daudi | - | 80 | 103 |
| | U937 | 116 | 85 | 120 |
| HDL only | Daudi | - | - | 97 |
| | U937 | - | - | 112 |
| CO only | Daudi | - | 124 | 106 |
| | U937 | 106 | 103 | 115 |

* Mixtures of CO with LDL and HDL were prepared as described in Table 2. Incorporation of [$^3$H]-thymidine was used as a measure of the effect of LDL and HDL, before and after treatment with CO, on the viability of Daudi and U937 cells. Cells were suspended in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, at a density of approximately 5.5 x $10^5$ cells per ml. Test materials were added in one-tenth the volume of that of the cell suspension. Culture medium alone was added to parallel cultures as controls. Control and treated cell suspensions were distributed in quadruplicate into 96-well tissue culture plates at 0.2 ml per well (approximately 1 x $10^5$ cells per well). After incubation for 6 hrs at 37°C in a humidified $CO_2$ incubator, 0.5 µCi of [$^3$H]-thymidine in a volume of 50 µl of culture medium were added to each well. After further incubation for 6 hrs, cells were collected on glass-fiber filters using a Skatron cell harvester. After drying, the filter discs were placed in vials with scintillant and counted on a liquid scintillation counter.

\+ Test materials were prepared as described in Table 2.

\- Not done.

pigs survived. Mice survived the intravenous injection of increasing amounts of enzyme-treated LDL, up to 20 times (the largest quantity tested) the amount that was lethal, on a weight basis, for rabbits. This result is consistent with the earlier finding that mice, unlike rabbits, fed for 12 weeks on a high cholesterol diet were refractory to challenge with cholesterol oxidase.

Enzyme treated LDL and HDL were tested for cytotoxicity for two human cell lines, B-lymphoblastoid cells (Daudi) (7) and macrophage-like histiocytes (U937) (8). Cholesterol-oxidase treated LDL proved toxic for both cell lines whereas cholesterol-oxidase treated HDL showed questionable toxicity for B-lymphoblastoid cells, and none for the histiocytes (Table 3). Neither enzyme nor lipoprotein alone was cytotoxic.

Enzyme treated LDL and HDL were also tested for cytotoxicity for bovine aortic endothelial cells isolated as described by Schwartz (1978) (9). Cells were plated into gelatin-coated 24-well tissue culture dishes and grown to confluence in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum, penicillin (10 μg/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). Confluent monolayers were washed twice with phosphate buffered saline, and cholesterol oxidase-treated LDL, untreated LDL, cholesterol oxidase-treated HDL, untreated HDL, cholesterol oxidase alone or phosphate buffered saline was added in a 1:10 mixture with the above growth medium. Cells were monitored microscopically at intervals up to 72 hrs for morphological and cytotoxic effects.

Untreated LDL had no visible effect on the cells during a 48-hr incubation period. In contrast, cells incubated with cholesterol oxidase-treated LDL underwent in 24 hrs a morphological change from the characteristic cuboidal cobblestone-like morphology characteristic of endothelial cells to an elongated morphology. A small percentage of the cells detached from the monolayer. By 48 hrs, over 75% of the cells were detached and lysed. Cells incubated with untreated HDL or cholesterol oxidase-treated HDL showed elongation by 24 hrs, but the monolayer remained intact throughout the 48-hr incubation period. Cholesterol oxidase alone was cytotoxic to the cells at a concentration of 10 μg/ml, but the effect required approximately twice as long as that produced by cholesterol oxidase-treated LDL.

The concept that a toxic agent may be important in the pathogenesis of atherosclerosis is not new. For example, Portman et al. (10) suggested that lysolecithin may function as the causal agent, and Vidaver et al. (11) have explored this hypothesis extensively. Imai et al. (12) found that cytotoxic autoxidation products of cholesterol can, by feeding, induce arterial damage in rabbits. Much subsequent work has led to the conclusion that dietary, spontaneously formed oxidation products of cholesterol, cannot be a primary factor in the genesis of atherosclerosis either in humans or in animals. Cholestenone is not present among these products. (For reviews, see Higley et al. (13) and Stehbens (14)) Recently, there has been much interest in the fact that endogenous peroxidation of lipids in LDL results in formation of oxidized LDL (15), and that LDL so modified, may be atherogenic. However, here the fatty acid chains in LDL are oxidized, and again, cholestenone has not been demonstrated to be present among the products.

If LDL-one functions in the initiation or development of atheromata, it is necessary to explain its origin. Cholesterol oxidase apparently is present in liver where it produces cholestenone as an intermediate in the conversion of cholesterol to cholestanol (16). The enzyme is also a product

of a number of bacteria (17), some of which occur in the large intestine where the enzyme functions in the first step in conversion of cholesterol to coprosterol. Cholesterol-oxidase producing bacteria, especially corynebacteria, are also known to occur at least occasionally among the human pharyngeal flora (18). Some proteins are absorbed through the gut (19-21), and cholesterol oxidase, if absorbed, could gain access to the bloodstream, and there convert LDL to LDL-one. In studying the induction of arterial lesions under a variety of conditions, Horsch and co-workers (22) found that germ-free rats on a high cholesterol peanut-oil diet did not develop aortic lesions, whereas ordinary rats on the same diet did, an observation that clearly supports the idea that microorganisms may be requisite for initiation of disease. In addition, in some humans, cholestenone may be carried by way of chylomicrons to the liver for VLDL synthesis where cholestenone might substitute for some of the cholesterol in VLDL. The suggested culpability of LDL-one is supported by the fact that small amounts of cholestenone are present in human sera (23).

The cholesterol of normal erythrocytes, and presumably that of other cell types, is not susceptible to the action of cholesterol oxidase, but in contrast, erythrocytes having membranes containing abnormally large amounts of cholesterol <u>are</u> susceptible to the enzyme (24). In our view, the lethal phenomenon described can be best explained by an abnormal vulnerability of cell membranes to damage eventuating from the action of the enzyme. Experiments that may support this hypothesis are in progress.

Our observations lead us to propose that several requirements must be satisfied in order for atherosclerosis to develop in man and animals. It appears that abnormally high levels of LDL are necessary but not sufficient for development of lesions. Among additional factors that may participate, we suggest that conversion of LDL to LDL-one and/or cholesterol oxidase itself, may function in pathogenesis. In addition, it may be necessary to have one or more types of cells that are susceptible to injury by LDL-one or by cholesterol oxidase. It would be of interest to test further the validity of these ideas.

ACKNOWLEDGEMENTS: We acknowledge with thanks helpful discussions with H. P. Bernheimer, M. R. J. Salton, H. J. Kayden and M. G. Traber. We are also indebted to M. G. Traber for providing LDL and HDL, and for determining that cholesterol was converted to cholestenone. This project was supported in part by BRSG S07 RR05399-23 awarded (to A.W.B.) by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, and by CA 37385 (to Y.K.Y.) from the National Cancer Institute, National Institutes of Health.

REFERENCES


1. Smith, A.G. and Brooks, C.J.W. (1976) J. Steroid Biochem. 7, 705-713.
2. Watanabe, Y., Ito, T. and Shiomo, M. (1985) Atherosclerosis 56, 71-79.
3. Imshenetskii, A.A. et al. (1982) Biol. Nauki (Moscow) 11, 52-55.
4. Lorincz, A.L., Jacoby, J.J. and Livingstone, H.M. (1948) Anesthesiology 9, 162-174.
5. Uwajima, T., Yagi, H., Nakamura, S. and Terada, O. (1973) Agr. Biol. Chem. 37, 2345-2350.
6. Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) J. Clin. Invest. 34, 1345-1553.
7. Klein, E. and Klein, G. (1968) Cancer Res. 28, 1300-1310.
8. Sundstrom, C. and Nilsson, K. (1976) Int. J. Cancer 17, 565-577.
9. Schwartz, S.M. (1978) In Vitro 14, 966-980.
10. Portman, O.W., Soltys, P., Alexander, M. and Osuga, T. (1970) J. Lipid Res. 11, 596-604.
11. Vidaver, G.A., Ting, A. and Lee, J.W. (1985) J. Theor. Biol. 115, 27-41.
12. Imai, H., Werthessen, N.T., Taylor, C.B. and Lee, K.T. (1976) Arch. Path. Lab. Med. 100, 565-572.
13. Higley, N.A. et al. (1986) Atherosclerosis 62, 91-104.
14. Stehbens, W.E. (1986) Prog. Cardiovascular Dis. 29, 107-128.
15. Steinbrecher, U.P., Parsatharathy, S., Leake, D.S., Witzum, J.L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. USA 81, 3883-3887.
16. Salen, G., Shefer, S. and Berginer, V.M. (1983) In The Metabolic Basis of Inherited Disease (J.B. Stanbury et al., eds.), Ed. 5, pp. 713-730 (McGraw-Hill, New York).
17. Kritchevsky, D. (1958) Cholesterol, John Wiley and Sons, Inc., New York, p. 132.
18. Linder, R. (1984) Biochem. Biophys. Acta 779, 423-435.
19. Walker, W.A., Isselbacher, K.J. and Bloch, K.J. (1972) Science 177, 608-610.
20. Hemings, W.A. (1978) Antigen absorption by the gut, University Park Press, Baltimore, MD.
21. Swarbrick, E.T., Stokes, C.R. and Soothill, J.F. (1979) Gut 20, 121-125.
22. Horsch, A.K. Kuhlmann, W.D., Bleyl, U. and Salomon, J.C. (1978) Res. Exp. Med. (Berlin) 173, 251-259.
23. Gray, M.F., Lawrie, T.D.V. and Brooks, C.J.W. (1971) Lipids 6, 836-843.
24. Lange, Y., Matthies, H. and Steck, T.L. (1984) Biochim. Biophys. Acta 769, 551-562.