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Oxidized low-density lipoprotein induces the production of interleukin-8 by endothelial cells

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Abstract **The concentration of interleukin-8 (IL-8) and RANTES was measured in culture supernatants of human EA.hy 926 endothelial cells incubated with oxidized low-density lipoproteins (LDL). Oxidized LDL induced a 3-fold increase in IL-8 production ($p < 0.01$), whereas RANTES was not detected. Native LDL did not stimulate IL-8 production. IL-8 production in oxidized-LDL-treated cells was mediated by reactive oxygen species, as it was partially inhibited by catalase and completely inhibited by glutathione peroxidase and *N*-acetylcysteine ($p < 0.01$).**

Key words: Atherosclerosis; Oxidized low-density lipoprotein; EA.hy 926 endothelial cell; Interleukin-8; RANTES; Reactive oxygen species

1. Introduction

Elevated plasma concentrations of low-density lipoproteins (LDL) are associated with accelerated atherogenesis [1,2]. The pathogenesis of an atherosclerotic plaque, first observed as a fatty streak, begins with the transport of lipoproteins into the artery wall, where oxidative modification of the LDL may occur due to the release of oxidative products by nearby cells [2–5]. Monocytes are recruited, matured in macrophages and then give rise to foam cells [1]. Proliferative smooth muscle cells, T lymphocytes and a few B lymphocytes are also found in atherosclerotic plaques. The phenotypes of T lymphocytes are predominantly CD 45 RO+ memory CD4+ and CD8+ subsets [6,7]. In response to antigen stimulation, these cells can induce the production of many inflammatory cytokines like interferon-γ (IFN-γ), tumor necrosis factor-α, (TNF-α) and interleukin-1 (IL-1). Although the paucity of neutrophils in atherosclerotic lesions has been documented, polymorphonuclear neutrophils (PMNs) may be implicated in the earliest phase of atherosclerosis [6–9].

The activation of endothelial cells by oxidized LDL (ox-LDL) may induce the expression of various inflammatory mediators, in particular of chemokines which modulate leucocyte adhesion and migration across the endothelium [2,3,10–12]. Minimally ox-LDL can induce the synthesis of the monocyte chemotactic protein-1 (MCP-1) which is chemotactic and activating for monocytes/macrophages [13,14]. T lymphocytes may be recruited by other chemokines.

Chemokines are low molecular mass cytokines (8–10 kDa) of which there are two subgroups: the C-C chemokine family – typified by MCP-1 and RANTES (regulated on activation normal T expressed and secreted) – and the C-X-C family – typified by interleukin-8 (IL-8) [11,12].

IL-8, produced by macrophage foam cells in human atheroma, may be involved in atherosclerosis [15]. Although initially identified as a neutrophil-specific cytokine, IL-8 also acts as a chemoattractant for T lymphocytes [12]. Attraction of neutrophils may depend on the IL-8 concentration gradient [16]. The β-chemokine RANTES selectively recruits and activates CD4+ memory T lymphocytes and monocytes but not neutrophils [11,12,17]. IL-8 and RANTES preferentially attract CD45RO T lymphocytes, which are the predominant type in atherosclerotic lesions [6,12,17].

IL-8 is a multifunctional chemokine involved in many biological processes, several of which may play roles in atherogenesis. Smooth muscle cell attraction and proliferation is implicated in atherosclerotic plaque formation and neovascularization appears in more advanced lesions and predisposes to intramural hemorrhage and plaque rupture [18]. As IL-8 is a potent mitogen and chemoattractant of vascular smooth muscle cells, it may be involved in smooth muscle cell invasion of the intima [19]. Its angiogenic capacity may contribute to the neovascularization associated with the atherosclerotic plaque [20,21].

EA.hy 926 endothelial cells are a reliable model for studying vascular inflammation, leucocyte-endothelial cell interactions and the metabolic effects of ox-LDL [22–25]. We investigated the effects of ox-LDL on RANTES and IL-8 production by these endothelial cells. Since reactive oxygen species (ROS) have been previously reported to upregulate IL-8 gene transcription and secretion in non-immune cells [26], we studied the effects of various antioxidants on the IL-8 production by endothelial cells incubated in the presence of ox-LDL.

2. Materials and methods

2.1. Chemicals

L-Glutamine, Dulbecco's modified Eagle's medium (DMEM), and trypsin-EDTA solution were obtained from Gibco. Foetal calf serum (FCS) was obtained from Boehringer, Mannheim. KBr and malondialdehyde (MDA) were obtained from Merck. Superoxide dismutase (SOD), glutathione peroxidase (Gpx) (from bovine erythrocytes), catalase (from bovine liver), *N*-acetylcysteine (NAC) and L-buthionine-*S,R*-sulphoximine (BSO) were from Sigma (France). Recombinant human TNF-α and TGF-β were obtained from PreproTech Inc. (Rocky Hill, NJ).

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Abbreviations: ox-LDL, oxidized low-density lipoproteins; IL-8, interleukin-8; RANTES, regulated on activation normal T expressed and secreted chemokine; SOD, superoxide dismutase; Gpx, glutathione peroxidase; NAC, *N*-acetylcysteine; BSO, L-buthionine-*S,R*-sulphoximine; PMNs, polymorphonuclear neutrophils; NF-kB, nuclear factor-kappaB

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2.2. Endothelial cell culture

The human EA.hy 926 endothelial cell line was obtained from Dr. C.-J.S. Edgell (University of North Carolina, USA) [27]. The cells were cultured in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and HAT (1000 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine). Factor VIII-antigen production was monitored.

2.3. LDL isolation and oxidation

LDL was isolated by sequential ultracentrifugation of human pooled plasma in the presence of EDTA according to Havel et al. [28]. Isolated LDL was dialysed for 24 h against a Tris-HCl buffer (0.01 M, pH 7.4) containing 1 mM EDTA, sterilised by filtration and stored at 4°C. The lipid components and the apolipoprotein B (apo B) content were determined and the absence of apolipoprotein A verified.

Before experiments, EDTA was removed by dialysis against phosphate-buffered saline (PBS, 10 mM, pH 7.4). LDL samples were adjusted to a final concentration of 2 mg apoB/ml with PBS. LDL was oxidized at 37°C in the presence of 5 μM $CuSO_4$ for up to 22 h and then dialysed against three changes of PBS for 24 h. LDL samples were filter-sterilised and incorporated in the culture medium. Filtration caused loss of 10% of the apo B and this was taken into account when adjusting LDL concentrations.

2.4. Oxidized LDL characterization

LDL oxidation was evaluated by assaying thiobarbituric acid reactive substances (TBARS) according to Yagi [29]. Although the TBARS content was 40–50 nmol/mg apo B before dialysis, it was reproducibly 5–8 nmol/mg apo B after extensive dialysis against three changes of PBS. The lipoperoxide concentration was around 1500 nmol/mg apo B, as determined by a kinetic application of the method of El Saadani et al. using cumene hydroperoxide as standard [30,31]. Electrophoretic mobility was characterized by the isolation of fractions A–D by HPLC analysis following the method of Vedie and coworkers adapted to the Waters chromatographic equipment [32]. Native LDL was 100% fraction A and ox-LDL 100% fraction C. No lipopolysaccharide (LPS) was detectable in the native LDL and ox-LDL preparations by the *Limulus* test technique, which can detect as little as 2 ng of LPS.

2.5. Incubation with oxidized LDL

Cells were diluted to 5×10^5 cell/ml, plated (200 μl) in 96-well plates and then allowed to reach confluence. Native LDL and ox-LDL (50, 100, 200 μg/ml), sterilized by filtration through 0.22 μm Millipore membranes, were added to the culture medium and incubated with endothelial cells at 37°C for 24 h. When used, SOD, Gpx and catalase were pre-incubated with the cells for 15 h. Cells were rinsed twice with DMEM and the culture medium replaced with fresh medium without enzyme containing native and ox-LDL (100 μg/ml). NAC and BSO were pre-incubated 2 h before adding LDL.

2.6. Regulation by TGF-β and TNF-α

Cells were diluted to 5×10^5 cell/ml, plated (200 μl) in 96-well plates and then allowed to reach confluence. Cells were rinsed twice with DMEM and then stimulated with TNF-α (10, 100, 1000 U/ml) for 24 h. When used, TGF-β (1, 10, 20 ng/ml) was pre-incubated with the cells for 20 h before adding TNF-α (100 U/ml).

2.7. Assays for cytokines

After incubation with LDL, supernatants were removed and assayed for IL-8 and RANTES using the ELISA IL-8 and RANTES kits, respectively, obtained from R&D Systems (Minneapolis, MN). All data are presented as means in pg/ml of duplicate samples determined using standard curves.

2.8. Statistical analysis

Analysis of variance (ANOVA) was used for comparing the means of several groups, with $p<0.01$.

3. Results

3.1. Effect of TGF-β on the production of IL-8 by TNF-α stimulated endothelial cells

Stimulation with TNF-α increased the production of IL-8 in a dose-dependent manner. The IL-8 concentration in culture media was 3843±164, 9737±312 and 14890±283 pg/ml following treatment with 10, 100 and 1000 IU/ml TNF-α, respectively. Treatment of EA.hy 926 endothelial cells with TGF-β (1, 10, 20 ng/ml) alone did not significantly change the level of IL-8 production. Pretreatment of EA.hy 926 endothelial cells with TGF-β prior to TNF-α (10 U/ml) stimulation significantly inhibited in a dose-dependent fashion the production of IL-8 (Fig. 1). In the presence of 10 ng/ml of TGF-β, the level of IL-8 in the culture supernatant was 1941 pg/ml, corresponding to 50% inhibition ($p<0.01$). Treatment of EA.hy 926 cells with TGF-β (up to 20 ng/ml) did not affect cell viability, as assessed by trypan blue staining (data not shown).

3.2. Oxidized LDL induce IL-8 production by EA.hy 926 endothelial cells

IL-8 and RANTES concentrations were measured in the supernatants of confluent cultures of EA.hy 926 cells incubated with copper ox-LDL for 4 and 24 h. No RANTES was produced whatever the concentration of native LDL or ox-LDL from 50 to 200 μg/ml. In contrast, IL-8 production was 3-fold higher by cells incubated for 24 h with ox-LDL than by control cells or cells incubated with native LDL (Fig. 2). IL-8 production was not significantly increased by 4 h incubation with either native LDL or ox-LDL. After 24 h incubation with ox-LDL, a dose-dependent increase in IL-8 production was observed up to 200 μg/ml of ox-LDL whereas no increase was found with native LDL (Fig. 3).

3.3. IL-8 production is free radical dependent in ox-LDL treated cells

To determine whether free radicals were involved in IL-8 production, we assessed the effect of various antioxidant en-

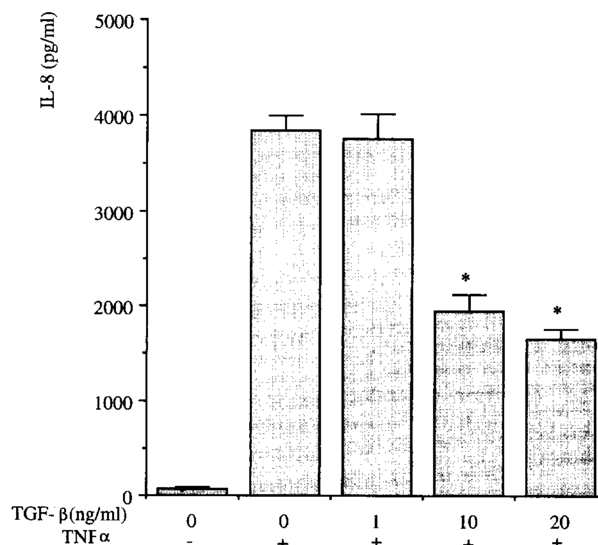

Fig. 1. Inhibition of IL-8 production by TGF-β in TNF-α-activated EA.hy 926 endothelial cells. EA.hy 926 cell monolayers on 96-well plates were pretreated with various concentrations of TGF-β (1, 10, 20 ng/ml) for 20 h at 37°C. After decanting the medium, cells were washed twice with DMEM, and then stimulated with TNF-α (10 U/ml) for 24 h. Culture supernatants were collected and assayed for IL-8 by ELISA assay. The results shown are the means±S.D. of one experiment carried out in duplicate which was representative of two. *$p<0.01$ vs. group receiving TNF-α (10 U/ml) but no TGF-β.

zymes: SOD, catalase and Gpx (Fig. 4). The level of IL-8 in the supernatant of cells incubated without LDL was 288 ± 15 pg/ml, that of cells incubated with native LDL being 256 ± 16 pg/ml. The basal IL-8 concentration was not modified by the presence of antioxidant enzymes. Ox-LDL-IL-8 induced secretion was completely inhibited by Gpx (100 U/ml) and moderately but significantly inhibited by catalase (600 U/ml). In contrast, the scavenging enzyme SOD (600 U/ml) did not prevent the increase in IL-8 production. Heat-inactivated enzymes were ineffective (data not shown) indicating that specific enzymatic activities were required for the inhibition of IL-8 production.

As Gpx was the most effective enzyme in preventing IL-8 production, we measured the cellular Gpx activity. The Gpx activity in EA.hy 926 cells (11.95 ± 6.7 IU/g) was increased 18-fold to 211.9 ± 9.9 IU/g by pre-incubation with Gpx. In addition, the glutathione precursor NAC at 10 mM also completely inhibited IL-8 production. The glutathione synthesis inhibitor BSO (0.1, 1 and 10 mM) did not increase IL-8 production.

4. Discussion

We have shown for the first time that ox-LDL induces the production of the chemotactic cytokine IL-8 by human EA.hy 926 endothelial cells. RANTES was not induced. We have also shown that IL-8 secretion in ox-LDL treated cells is mediated by ROS.

Most of the information needed for cell (lymphocytes, endothelial cells, neutrophils) activation is communicated by cytokines. Neutrophil migration, adhesion to vascular endothelial cells, protease release and ROS production and lymphocyte T cell attraction is mediated by IL-8 [11,12]. IL-8 production is stimulated by IL-1β, IL-1α, TNF-α and LPS in vascular endothelial cells and this enhances the chemotaxis of neutrophils and T cells [11,12]. We have shown that IL-8 secretion can also be induced by oxidized-lipid mediators. Incubation of endothelial cells with ox-LDL resulted in a 3-fold increase in IL-8 production whereas native LDL did not stimulate IL-8 release.

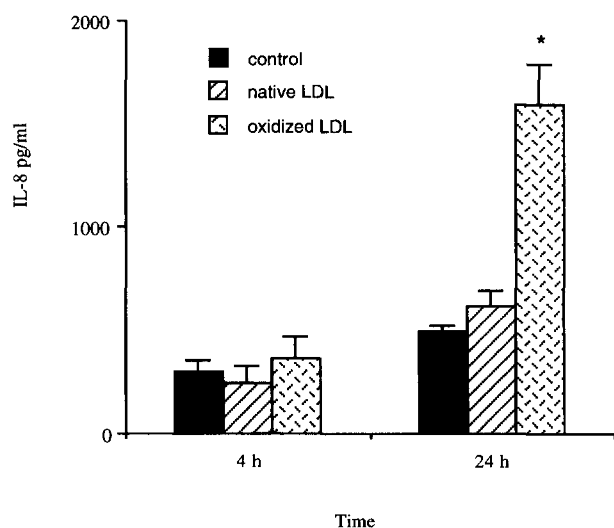

Fig. 2. IL-8 accumulation in supernatants of endothelial cell cultures. EA.hy 926 cell monolayers on 96-well plates were incubated with native and oxidized LDL (100 μg/ml) and supernatants were assayed for IL-8 after 4 and 24 h. Results are expressed as mean IL8 production ± S.E.M., $n = 3$. $^{*}p < 0.01$ relative to control cells.

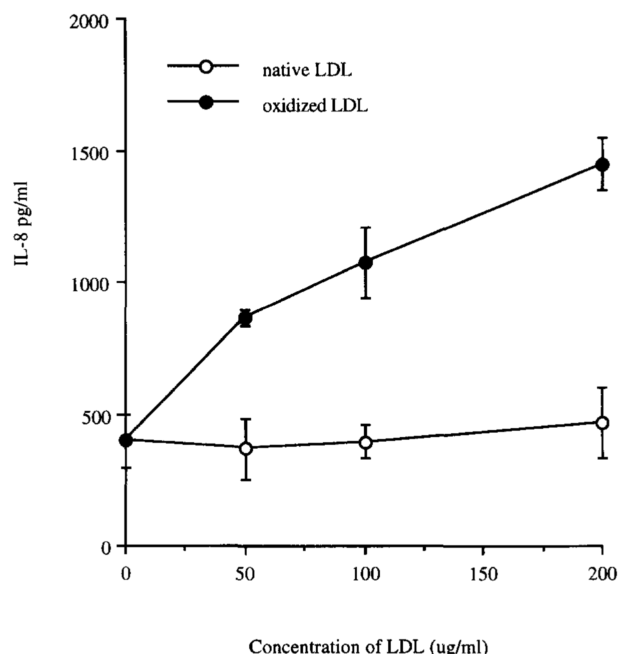

Fig. 3. Dose effect curves of oxidized LDL on IL-8 production by endothelial cells. EA.hy 926 cell monolayers on 96-well plates were incubated with native and oxidized LDL at the following concentrations: 50, 100, 200 μg/ml. IL-8 concentrations were determined after 24 h. Results are expressed as mean IL-8 production ± S.E.M., $n = 3$.

In our copper ox-LDL preparations, the apo B was modified but 90% of the TBARS were water-soluble material lost after extensive dialysis against PBS as previously described [33]. In contrast, the lipoperoxide content was higher than those reported by some but not all workers [34,35]. However, ox-LDL composition depends on the conditions of copper oxidation: the ratio of Cu^{2+}/LDL, medium and temperature [36]. In addition, the differences in reported levels of lipid hydroperoxides may arise from the various techniques used or different reactivities of various classes of hydroperoxide [35].

The basal levels of IL-8 secretion by EA.hy 926 cells were not higher than those described for HUVEC. Moreover, EA.hy 926 endothelial cells respond to TNF-α like primary cells [37,38]. They are induced by TNF-α to produce IL-8 in a dose-dependent manner and 10 ng/ml TGF-β inhibits TNF-α-induced IL-8 production by 50% [38,39].

Our results also demonstrate the involvement of ROS in IL-8 production by endothelial cells induced by ox-LDL. The metal ion-catalyzed reaction between superoxide and hydrogen peroxide (H_2O_2) gives rise to the highly reactive hydroxyl radical $OH^{\bullet}$. Catalase and SOD inhibit cytokine synthesis induced by hypoxia and H_2O_2 [40,41]. Exogenous Cu/Zn-SOD has been shown to penetrate cells [40,42,43]. However, SOD did not prevent ox-LDL induced IL-8 production in our study. This suggests that superoxide anions are not involved in this phenomenon or are inactivated by other means (spontaneous dismutation or chemical scavengers). In contrast, a moderate but significant inhibition of ox-LDL induced IL-8 production was observed with catalase, implicating H_2O_2 [44]. Uptake of catalase by endothelial cells has been demonstrated but it may also act extracellularly at plasma membrane sites

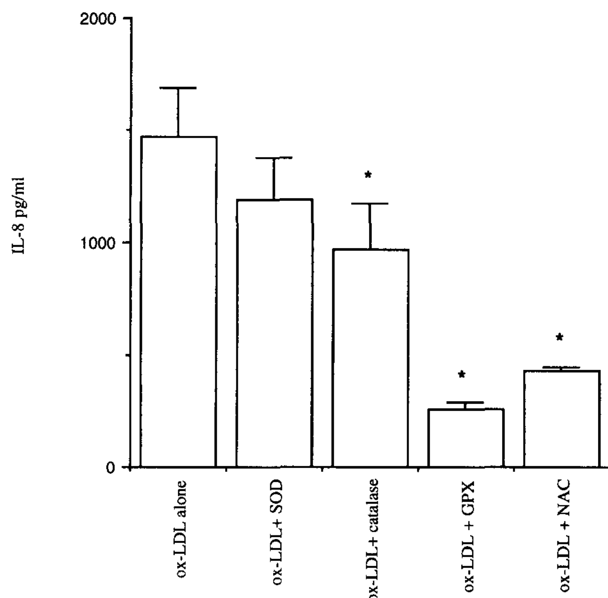

Fig. 4. Effect of antioxidants on oxidized LDL-induced IL-8 synthesis by endothelial cells. EA.hy 926 cell monolayers on 96-well plates were pre-incubated overnight with superoxide dismutase (SOD, 600 U/ml), catalase (600 U/ml), and glutathione peroxidase (GPx, 100 U/ml). After decanting the medium, cells were washed twice with DMEM before adding native and oxidized LDL (100 μg/ml). *N*-Acetylcysteine (NAC) was pre-incubated 2 h before LDL addition. IL-8 concentrations were determined after 24 h of incubation. Results are expressed as mean IL-8 production ± S.E.M., $n = 2$. *$p < 0.01$ relative to control cells.

[43]. The removal of H_2O_2 may prevent the formation of the initiating hydroxyl species OH˙ [44].

Pre-incubation with Gpx completely inhibited IL-8 production. The superiority of Gpx as compared to catalase suggested that the reduction of other peroxides over H_2O_2 might be involved [44]. These peroxides may be lipid hydroperoxides originating from ox-LDL or those formed from cellular lipids. After pre-incubation with Gpx, the cellular Gpx activity was 18-fold increased compared to control cells. Gpx may be endocytosed or be absorbed by the outer leaflet of the plasma membrane to antagonize the initiation of lipoperoxidation induced by hydroperoxides [45]. Glutathione is the specific substrate for Gpx and in the outer leaflet of the membrane, glutathione may originate from supernatants where thiols tend to liberate it from mixed disulfides with proteins [46]. NAC is a GSH precursor and has been reported to increase intracellular GSH levels in many cultured cells by promoting cysteine uptake [47,48]. Its inhibitory effect on IL-8 production indicates the importance of the glutathione redox cycle which counteracts lipoperoxidation and the involvement of lipoperoxy radicals. An inhibitory effect of Gpx on cytokine synthesis induced by hypoxia has been described by Ala et al. [40].

One possible mechanism for the ox-LDL-induced IL-8 secretion is alteration of the redox status of cells. Nuclear factor-kappaB (NF-kB) is a DNA-binding protein involved in the transcriptional activation of a variety of genes (TNF-α, IL-1, IL-6, MCP-1) [11,13,14] and its activation depends on the cell redox status [47]. NAC inhibits NF-kB activation, which has also been associated with the GSH content of cells [47,49,50]. The IL-8 gene contains NF-kB binding sites which can fix activated NF-kB and induce its transcription [11]. In this study, the inhibitory effects of NAC and Gpx indicate a possible inhibition of NF-kB activation. However, BSO did not increase basal or ox-LDL-induced IL-8 production. Possibly, as reported by Schultze and co-workers, oxidants can regulate, but are not sufficient to initiate NF-kB activation. This NF-kB activation may require additional signalling events such as protein tyrosine phosphorylation [51].

We did not detect RANTES in our culture supernatants, even at high ox-LDL concentrations. Both cytokine genes contain NF-kB binding sites. However, the mechanisms which control RANTES expression and secretion are different and may need costimulatory signals such as INF-γ [11,17].

The release of numerous cytokines causes the development of a network of activated macrophages, smooth muscle cells, T cells, and endothelial cells and leads to progression of the atherosclerotic lesion to a more advanced, complicated lesion [2,3,52]. The identification and characterization of genes involved in the cross-talk may help decipher the mechanisms of atherogenesis [52]. Secretion of IL-8 may: (i) attract CD45RO lymphocytes; (ii) induce smooth muscle cell proliferation; (iii) induce angiogenesis, giving rise to advanced atherosclerosis [12,19,20]. IL-8 may also attract and activate PMNs, although their paucity in the atherosclerotic plaque has been documented [7]. In some models, the formation of intimal thickening is characterized by the infiltration of PMNs, monocytes and lymphocytes. PMNs are present during the early phase of lesion development and their disappearance from the intima may result from their phagocytosis by macrophages and their migration toward the adventia [9]. Understanding the mechanism which regulates IL-8 secretion may help explain the clinical significance of the increase of ox-LDL in atherosclerosis. The exact mechanism of IL-8 secretion and its implications for patients with coronary artery disease are under study in our laboratory.

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