Effect of cholesterol and 7-β hydroxycholesterol on glutathione status and nitric oxide production in murine peritoneal macrophages

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Present study was conducted to observe the effect of cholesterol and oxidized cholesterol (7β-hydroxycholesterol, 7β-OH) on the nitric oxide (NO) production and the redox ratio by lipopolysaccharide-stimulated macrophages. Dose-dependent decrease in NO levels was seen with both cholesterol and 7β-OH at different incubation intervals (6, 12, 18, 24 hr) and concentrations (2.5, 5, 7.5 μg/ml). On comparison, a significant decrease in the NO was observed at 24 hr interval in 7β-OH exposed cells with all respective concentrations of cholesterol. Incubation with 7β-OH also resulted in significant increase in levels of oxidized glutathione (GSSG) and decrease in reduced glutathione (GSH), while cholesterol showed no effect on GSSG levels. Moreover, GSH levels were lowered only at highest concentration (7.5 μg/ml), and at longer incubation intervals (18, 24 hr) with cholesterol exposure. This altered the redox status in both cholesterol/7β-OH treated macrophages. Increased redox ratio and decreased NO levels indicated increased oxidative stress and decreased vasodilation by 7β-OH compared to cholesterol.

Keywords: Cholesterol, Glutathione status, 7β-Hydroxycholesterol, Murine peritoneal macrophages, Nitric oxide

Evidences exist that implicates free radical damage via oxidized lipids as a key step in atherosclerosis. Of the cells visible in the atherosclerosis, foam cells attract attention and it seems that most foam cells are macrophages by morphology. It is of further interest that macrophages take up low density lipoprotein (LDL) very slowly and do not change to foam cells unless LDL is modified or oxidized. Oxysterols are biologically active molecules generated during the oxidation of the LDL. Several of these oxysterols are found in the macrophage derived foam cells. However, sterols oxygenated at the 7th position viz. 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7KC), have been shown to predominate in copper-oxidized LDL as well as human atherosclerosis plaques.

One mechanism that the macrophages use to exert their cytostatic and cytotoxic effects on the target cells is by releasing nitric oxide, NO. NO is a cellular mediator and has many biological functions including macrophages mediated cytotoxicity, neurotransmission and smooth muscle relaxation. Endothelium derived NO is a potent endogenous nitrovasodilator and plays a major role in regulating the vascular tone.

Endothelium dependent relaxations are impaired in animals with experimentally induced atherosclerosis, which has been linked to a decreased biological activity of NO (Ref. 9). Moreover, the lack of NO results in increased adhesion of leukocytes, this in turn can be responsible for a number of pathophysiological conditions like generation of superoxide anion (O2-), oxidation of LDL and foam cell formation resulting in intimal thickening.

Another major endogenous protective system is the glutathione redox cycle. Glutathione (GSH) an essential tripeptide (L-glutamyl-L-cysteinyl-glycine), is the major non-protein thiol and plays an important role against oxidative stress and in the detoxification of many electrophiles. By acting as the substrate for glutathione peroxidase, it mediates destruction of H2O2 and hydroperoxides. Activated macrophages

Abbreviations
7β-OH—7β-hydroxycholesterol; ROS—reactive oxygen species; FCS—fetal calf serum; LPS—lipopolysaccharide; PBS—phosphate buffered saline; ox-LDL—oxidized low density lipoprotein; GSH-reduced glutathione; GSSG-oxidized glutathione; TCA—tricarboxylic acid; NOS—nitric oxide synthase; NO—nitric oxide; MTT—3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
release a large amount of ROS. GSH can easily detoxify them and protect against cell injury. Oxysterols are known to generate oxidative stress and depletion of cellular GSH has been considered a major contributory factor towards atherosclerosis.

In the present study, changes in the cellular redox ratio (oxidized glutathione/reduced glutathione, GSSG/GSH) and NO production in mouse peritoneal macrophages subjected to varying levels of cholesterol and 7β-OH for different intervals was studied.

Materials and Methods

For the present studies female Balb/c mice (3-4 months old) were obtained from the Central Animal House, Panjab University, Chandigarh. All the animals were fed on standard pellet diet and had free access to drinking water.

Dulbecco’s modified eagle medium, DMEM (with L-glutamine and without phenol red), phosphate buffered saline (Ca²⁺, Mg²⁺ free), fungizone, sodium bicarbonate and thioglycolate broth were obtained from HiMedia Lab Pvt Ltd, Bombay. 7β-OH cholesterol, trypsin blue, FCS, LPS were from Sigma, USA. Rest all other chemicals used in the present study were of analytical grade obtained from Indian labs.

Macrophage culture—Macrophages were cultured as described previously. Briefly, mice (4-5 in each group) injected intra-peritoneally with 2 ml of 3% (w/v) thioglycolate (TG) were sacrificed on the 5th day by cervical dislocation. Peritoneal cells were harvested by lavage and washed twice with ice-cold PBS. The cells were then resuspended in Modified Eagle’s medium without phenol red containing 10mM HEPES; 2g/l sodium bicarbonate; 10mg/l, gentamycin; 10%, FCS and 1.25 mg/l, fungizone. L-arginine (2mM) was added in the media for NO estimation. The cell concentration was adjusted to 1 million per ml using haemocytometer. The cells were dispensed into wells of tissue-culture plate and incubated for 2 hr at 37°C for cell adherence. Thereafter, the non-adherent cells were removed by flicking the plate.

The cells were incubated with varying concentration of cholesterol and 7β-OH (2.5, 5, 7.5 μg/ml) for different incubation intervals (6, 12, 18, 24 hr) in humidified condition of CO₂ (5%) + air (95%) at 37°C. Stock solutions of cholesterol and 7β-OH were prepared in ethanol and care was taken that the final ethanol concentration did not exceed 0.1% in wells. Concentrations of 7β-OH used in the present study were in the range of those measured in plasma from hypercholesterolemic patients and those found in atherosclerotic plaques. Viability of cells was also not affected at these treatments. LPS was added in each well at a concentration of 50 μg/ml. At the end of the incubation interval, 100μl of culture supernatant was taken for NO estimation. The cells were harvested in ice-cold PBS and washed two times with PBS to obtain the cell pellet.

Analysis of NO production—NO production was assessed by measuring nitrite, a stable metabolic product of NO, in macrophage culture supernatant by the method of Raddassi et al. In brief, 100μl Griess reagent (1:1 v/v) mixture of 0.1% N-[1-naphthyl] ethylenediamine dihydrochloride in double distilled water and 1% sulfanilamide in 2.5% ortho-phosphoric acid solution was added to 100 μl of the culture supernatant in ELISA strips. The plate was kept in dark for 10 min and the pink colour so obtained was read at 540 nm on a scanning multiwell spectrophotometer (ELISA reader, STAT fax 325).

The amount of nitrate produced in each well was determined by a standard curve prepared by using sodium nitrate. Results were expressed as mean ± SEM of 4 observations (nmole of nitrite accumulated in each well).

Estimation of oxidized, reduced glutathione and redox ratio in macrophages—The cell pellet was lysed in 0.1 M phosphate-EDTA buffer (pH 8.0) containing 0.1% triton X-100 and then protein was precipitated using 5% tricarboxylic acid (TCA). The lysate was mixed well and supernatant was used for estimation of total and oxidized glutathione, which were quantitated by using o-phthalaldehyde (OPT) as fluorescent reagent. This method was based on the reaction of GSH at pH 8 and GSSG at pH 12. GSH was complexed to N-ethylmaleimide (NEM) to prevent interference of GSH with the measurement of GSSG. GSH levels were obtained by subtracting the levels of GSSG from total glutathione levels. Cellular protein was determined by the method of Lowry et al. The results were expressed as nmole of GSSG/GSH per mg cell protein.

Statistical analysis—Results are given as mean ± SEM of quadruplicates. The statistical significance of differences between control cells and cells exposed to different concentrations of cholesterol/7β-OH at various incubation intervals was estimated by means of Student’s t test for unpaired values.
Results

Nitric oxide synthetase activity—The effect of cholesterol and 7β-OH on NO levels is shown in Fig. 1. Control as well as cholesterol/7β-OH treatments at various concentrations showed a time dependent increase in A540. Decrease in NO was observed with both cholesterol/7β-OH at each incubation interval, but this decrease was more significant only at higher concentrations viz., 7.5 µg/ml and longer incubation intervals (18 and 24 hr).

While comparing the effect of cholesterol and 7β-OH, no change was observed at earlier incubation intervals with increasing concentrations of cholesterol/7β-OH. However, a significant decrease in NO was observed at 24 hr interval in 7β-OH exposed cells with all respective concentrations of cholesterol.

Oxidized and reduced glutathione levels—Effect of cholesterol and 7β-OH on oxidized and reduced glutathione levels in the cell lysates of murine peritoneal macrophages cultured at different incubation intervals has been shown in Fig. 2 (i, ii). The cells subjected to cholesterol, showed no change in GSSG levels either with increasing concentration or incubation interval. With 7β-OH, a significant increase was observed at higher doses viz., 5 and 7.5 µg/ml at each incubation interval.

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Fig. 1—Effect of cholesterol and 7β-hydroxycholesterol on nitric oxide production [Values are Mean ± SEM of quadruplicates.*P < 0.05 and **P < 0.01 represents the comparison between control (untreated) cells and the cells exposed to cholesterol/7β-OH for each incubation interval. * P < 0.05, ** P < 0.01 and *** P < 0.001 represents the comparison between the cells at incubation interval of 6 hr and other intervals. * P < 0.05 represents the comparison between cells exposed to cholesterol and 7β-OH].

Fig. 2—Effect of cholesterol and 7β-OH on (i) GSSG (ii) GSH and (iii) redox ratio (GSSG/GSH). [Values are Mean ± SEM of quadruplicates.*P < 0.05, **P < 0.01 and ***P < 0.001 represents the comparison between control (untreated) cells and the cells exposed to cholesterol/7β-OH for each incubation interval. # P < 0.05, ## P < 0.01 and ### P < 0.001 represents the comparison between the cells at incubation interval of 6 hr and other intervals. * P < 0.05, ** P < 0.01 and *** P < 0.001 represents the comparison between cells exposed to cholesterol and 7β-OH].
Increase in GSSG was observed at all intervals in a dose-dependent manner in the cells exposed to 7β-OH. 7β-OH showed significantly increased GSSG levels at 5 and 7.5 μg/ml at 6 hr and at all concentrations at other incubation intervals, compared to the respective concentration and incubation intervals of cholesterol exposed cells.

No change in GSH was observed with progressive incubation interval in cholesterol exposed cells, but a significant decrease in GSH levels was observed at higher incubation intervals (18 and 24 hr), in the cells exposed to higher concentration of 7.5 μg/ml of cholesterol. In 7β-OH exposed cells, decreased GSH levels were observed at 7.5 μg/ml at 6 and 12 hr and at all concentrations at longer durations. Thus, the decline in GSH levels was more in 7β-OH exposed cells as compared to the respective cholesterol concentrations. The cellular redox ratio was determined by using the intracellular levels of GSSG and GSH and the data has been shown in Fig. 2(iii).

Discussion

There is substantial evidence that oxidative injury plays a major role in atherosclerosis. Glutathione is a well-known intracellular antioxidant and the ratio of oxidized and reduced glutathione (GSSG/GSH) is an accurate, specific and sensitive way of evaluating oxidative stress\(^2\). In the present study, both cholesterol and 7β-OH increased the redox ratio of cells at longer duration (18, 24 hr) and higher concentrations (5 and 7.5 μg/ml), which revealed that, prolonged incubation exposes the cells to higher oxidative stress. While comparing the effects of cholesterol and 7β-OH, the cells exposed to 7β-OH showed significantly higher ratio, which indicated that oxysterols were mainly responsible for the increased oxidative stress seen in atherosclerotic lesion.

Macrophages are able to produce the radical NO in response to many inflammatory stimuli like LPS and IFN-γ, which mediates the vasodilator response. In the present study, LPS at 50μg/ml concentrations stimulated the release of NO. It has been seen that bacterial LPS can increase both blood NO concentration in vivo and production of NO by macrophages in vitro\(^3\). However, it was observed that 7β-OH as well as cholesterol caused inhibition of NOS activity in a dose-dependent manner. A significant decrease in NO was also observed at 24 hr interval in 7β-OH exposed cells, compared with all respective concentrations of cholesterol which indicates that oxysterols like 7β-OH are more potent factors responsible for decreasing NO production rather than free cholesterol. A time dependent increase in the nitrite levels was observed in both cholesterol and 7β-OH as well as in the control cells. This might be due to accumulation of nitrite in the culture media.

The oxysterol-induced inhibition of NOS might contribute to the development of atherosclerosis by reducing the antiatherogenic properties of NO. Appearance of macrophages within the atherosclerotic lesions has been supposed to be due to increased recruitment of the circulating monocytes, via NF-κB dependent expression of vascular endothelial cell adhesion molecule (VCAM-1), macrophage colony stimulating factor (M-CSF) and monocyte chemoattractant protein (MCP-1). NO has been shown to inhibit VCAM-1\(^4\), M-CSF\(^25\) and MCP-1\(^26\) expression by inhibiting NF-κB activation. The development of atherosclerotic lesions as a consequence of chronic inhibition of NO production by N-nitro-L-arginine methyl ester (L-NAME), an inhibitor of both inducible and constitutive NOS, has been reported\(^27\). It has been recently found that ox-LDL and TNF-α increased the expression of adhesion molecules on endothelial cells through NF-κB activation and that this stimulus is inhibited by the presence of compounds with radical scavenging activity\(^28\). This again shows that cholesterol and largely 7β-OH, which diminish the cellular pool of GSH, can increase the adhesion molecules and make conditions pro-atherogenic. Since NO causes vasodilatation, decrease in its levels can further aggravate the problem.

Several hypotheses have been put forward to explain the decline in NO production. While some are of the opinion that this is through a direct inhibition of NOS activity by the lipid moiety of oxidized LDL\(^29\), others showed that Ox-LDL had no direct inhibitory effect on NOS activity but affects its transcription\(^30\). Another possible explanation is that oxysterols become incorporated in the membrane, thus, blocking the signal transduction pathway of LPS. Smith and Johnson\(^31\) have shown that oxysterols incorporated into cell membranes can inhibit many membrane related functions, some others suggest that oxysterols oxidized at the 7th position such as 7 ketocholesterol inhibit NO by activating protein kinase C\(^32\).

The glutathione levels and nitric oxide production seems to be linked. Decrease in aortic total glutathione (GSH) levels in hypercholesterolaemia is
related to the impairment of relaxation to acetylcholine and exogenous NO. Increasing GSH significantly enhanced NO-induced relaxation in aorta from both hypercholesterolaemic and normal rabbits. These data suggest that other factors, perhaps related to the long-term decrease in GSH levels, are responsible for reduced NO bioactivity in hypercholesterolaemia. In another study, hypercholesterolaemia decrease tissue GSH, attenuate the vasorelaxation response and potentiate peroxynitrite (ONOO−) induced vascular tissue injury.

In conclusion, 7β-OH at low concentrations caused significant decrease in glutathione levels and NO production compared to cholesterol and might play an important role in the development of atherosclerotic lesions by increasing oxidative stress and decreasing the activity of the enzyme NOS.

References


