Indian Journal of Biochemistry & Biophysics
Vol. 41, February 2004, pp. 14-19

Effect of cholesterol and 7β-hydroxycholesterol on glutathione status and expression of Hsp70 in cultured murine peritoneal macrophages

M P Bansal* and Sonia Shalini

Department of Biophysics, Panjab University, Chandigarh-160 014, India

Received 18 July 2003; revised 24 December 2003

Using cultured murine peritoneal macrophages, the change in redox ratio (oxidized/reduced glutathione) was studied at different incubation intervals (6, 12, 18 and 24 hr) with different concentrations (2.5, 5 and 7.5 μg/ml) of cholesterol and 7β-hydroxycholesterol (7β-OH), using fluorimeter. The changes in the levels of heat shock protein, hsp70 was determined using ELISA. Both cholesterol/7β-OH caused a decrease in hsp70 protein levels at all the incubation intervals in dose dependent manner but the decrease was significantly higher with 7β-OH. Treatment with 7β-OH also resulted in significantly increased levels of oxidized glutathione (GSSG) and decreased reduced glutathione (GSH) while cholesterol showed no effect on GSSG levels. Moreover, GSH levels were lowered only at the highest concentration (7.5 μg/ml) at longer incubation intervals (18 and 24 hr) with cholesterol exposure. This altered the redox status in both cholesterol/7β-OH treated macrophages. These results suggest that cholesterol and more likely 7β-OH may exert their pro-atherogenic effects by lowering hsp70 protein production and inhibiting glutathione synthesis by macrophages present in the arterial wall.

Key words: Cholesterol, 7-β hydroxycholesterol, glutathione status, hsp70, murine peritoneal macrophages

Cholesterol and oxidized low-density lipoproteins (Ox-LDL) have been associated with the genesis of atherosclerosis. Ox-LDL is a complex mixture of lipid hydroperoxides, aldehydes and oxidized cholesterol, also called oxysterol[1]. Oxysterols are biologically active molecules generated during the oxidation of LDL. The 7-oxygenated sterols, 7β-hydroxycholesterol (7β-OH) and 7-ketocholesterol have been shown to induce the expression of adhesion molecules on the endothelial cells and hence are believed to be involved in the adhesion of monocytes and lymphocytes during the formation of atherosclerotic lesions[2]. Atherosclerotic lesion is well known to have macrophages also which are sources of foam cells in the area[3,4]. One of the potentially atherogenic properties of Ox-LDL is its cytotoxicity to the cells of artery wall[5,6]. It is suggested that 7β-OH and 7-ketocholesterol, and not the cholesterol are responsible for the ability of Ox-LDL to reduce the endothelium-dependent relaxation of isolated rabbit aorta[7].

Mammalian cells have evolved protective mechanisms to minimize the injurious events that result from toxic chemicals and normal oxidative products of cellular metabolism. A major endogenous protective system is the glutathione redox cycle[8]. Glutathione is present in high concentrations as reduced glutathione (GSH) in most mammalian cells (generally in millimolar range), with minor fractions being oxidized glutathione (GSSG), mixed disulphides of GSH and other cellular thiols and thioesters[9]. It is an important water-phase antioxidant and essential co-factor for antioxidant enzymes[10]. Exposure of macrophages to inflammatory stimuli results in the production of superoxide anion (O_2^-) and H_2O_2, the reactive oxygen species (ROS), which play an important role in the cell's microbicidal and tumouricidal activities[11,12]. GSH can effectively detoxify ROS in the presence of superoxide dismutase (SOD)[13,14] enzyme and protect against oxidative damage.

Besides glutathione, the cells facing toxic stimuli rapidly and preferentially synthesize a family of cytoprotective proteins known as heat shock proteins (hsps)[15], of which the hsp70 family functions as a molecular chaperone to protect the cells from

*Corresponding author
E-mail: mpbansal@pu.ac.in
Tel: 0172-2534120; Fax: 0172-2534118

Abbreviations: 7β-OH, 7β-hydroxycholesterol; hsp, heat shock protein; ROS, reactive oxygen species; BSO, buthionine-(S,R)-sulfoximine; hsf-1, heat shock transcription factor 1; FCS, fetal calf serum; LPS, lipopolysaccharide; PMSF, phenyl methyl sulfonyl fluoride; OTC, L- 2- oxothiazolidine-4-carboxylate; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; ox-LDL, oxidized low density lipoprotein; GSH, reduced glutathione; GSSG, oxidized glutathione; TCA, tricarboxylic acid.

environmental-stress damage, by binding to denatured proteins, reshaping of damaged proteins and dissociation of protein aggregates[16]. This stress response represents one of the basic mechanisms of cellular defense. Cytotoxicity of oxysterols has been reported and endogenous hsp70 supplementation is shown to provide oxidative protection to myocytes[17], fibroblasts[18] and arterial smooth muscle cells[19]. Both glutathione and hsp70 are involved in cytoprotection. Earlier the effect of Ox-LDL on these parameters has been reported[20]. In the present study, the effect of cholesterol and 7β-OH has been compared by determining the changes in the glutathione redox (GSSG/GSH) ratio and hsp expression at various incubation intervals in the cultured murine peritoneal macrophages.

Materials and Methods

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 (Ca^{2+}, Mg^{2+} free), fungizone, sodium bicarbonate and thioglycolate broth were obtained from Himedia Laboratories Pvt Ltd, Bombay. 7β-OH, cholesterol, trypan blue, fetal calf serum (FCS) and lipopolysaccharide (LPS) were from Sigma-Aldrich, USA. All other chemicals used were of analytical grade obtained from Indian manufacturers.

Macrophage culture

Mice (4-5 in each group) injected intra-peritoneally with 2 ml of 3% (w/v) thioglycolate (TG) each, were sacrificed on the fifth day by cervical dislocation. Peritoneal cells were harvested by lavage with ice-cold PBS and centrifuged at 2000 rpm for 15 min in sterile tubes. RBCs were lysed by ice-cold NH_4Cl solution and then the pellet was washed repeatedly with PBS to remove any traces of NH_4Cl. The cells were then suspended in 1 ml of media and cell concentration was adjusted to 1 million per ml using haemocytometer. To each well of 96-well tissue-culture plate, 100 µl of cell suspension was added. This was incubated for 2 hr at 37°C in humidified atmosphere of 5% CO_2+95% air for cell adherence. Thereafter, the non-adherent cells were removed by flicking the plate on filter paper.

The cells were then dispensed in 96-well tissue culture plate and incubated with varying concentrations of cholesterol and 7β-OH (2.5, 5 and 7.5 µg/ml) for different incubation intervals (6, 12, 18 and 24 hr) in humidified condition of 5% CO_2 +95% air at 37°C. The 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[21]. At the end of incubation period, the cells were harvested and washed two times with PBS.

Redox ratio in macrophages

The pellet was lysed in 0.1 *M* phosphate-EDTA buffer (*p*H 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*-phthaldehyde (OPT) as fluorescent reagent[22]. This method is based on the reaction of GSH at *p*H 8 and GSSG at *p*H 12. GSH is 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*[23]. The results were expressed as nmoles of GSSG/GSH per mg cell protein.

Determination of hsp70 protein levels using ELISA

The cells were harvested from the culture plate and centrifuged to obtain a pellet, which was suspended in lysis buffer (0.5 *M* tris-HCl, *p*H 6.7; 0.5% SDS and 1 m*M* PMSF). The cells were properly lysed by rapid freeze-thaw cycles and lysate was then centrifuged and the supernatant used for the estimation of protein[23]. About 10 µg of lysate protein was coated on each well of ELISA strip. The unbound space was blocked by incubation with 1% BSA in 0.1 *M* PBS for 1 hr and washed with 200 µl of PBS containing 0.05% (v/v) tween-20. Thereafter, 100 µl primary monoclonal antihsp70 antibody was added (at a dilution of 1:1000 in PBS containing 0.05% tween-20 and 1% BSA) and the plate kept at 37°C for 2 hr. The plate was again washed with PBS containing 0.05% (v/v) tween-20, followed by the addition of biotin labeled antimouse IgG as secondary antibody (1: 500 dilution) and incubation for 2 hr at 37° C. Then streptavidin labeled peroxidase was added and again incubated for 2 hr at 37° C. Coloured reaction was developed by adding the substrate, 2,2′-azino-di-(3-ethyl-benzothiozolin sulphonic acid) [ABTS]. The

plate was kept in the dark for 30 min and the coloured product was quantitated at 405 nm using an ELISA reader (Stat Fax 325).

Statistical analysis

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

Results

Oxidized and reduced glutathione levels

The effect of cholesterol and 7β-OH on oxidized glutathione levels in the cell lysates of murine peritoneal macrophages cultured at different incubation intervals is shown in Table 1. 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 18 hr and at all the concentrations at 24 hr. 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 levels was observed with progressive incubation interval in cholesterol-exposed cells. However, a significant decrease was observed at higher incubation intervals (18 and 24 hr) in the cells exposed to higher concentration (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 are shown in Table 1.

Heat shock protein 70 (hsp70) levels

The changes in the levels of hsp70 in peritoneal macrophages exposed to cholesterol/7β-OH was studied, using ELISA and the data is shown in Table 2. In control, no change in hsp70 levels was observed with increasing incubation intervals. With cholesterol, significant increase was observed at all concentrations at various intervals. However, with 7β-OH, a significant decrease was seen with 7.5 μg/ml concentrations at 18 and 24 hr. Cholesterol showed a significant decrease in hsp70 only at 7.5 μg/ml concentrations at all intervals, compared to the respective controls, whereas 7β-OH showed significant decrease even at 5 μg/ml at all intervals. On comparison, the cells exposed to 7β-OH showed significantly reduced hsp70 levels with 5 and 7.5 μg/ml concentration at 12, 18 and 24 hr, compared to the respective concentration and intervals of incubation of cholesterol-exposed cells.

Discussion

Activated macrophages produce ROS such as $O_2^{-\cdot}$ and H_2O_2. Since the cells resist these ROS and subsequent toxic by-products, they might have potent defense systems to minimize the deleterious effects of these insulting agents. Treatment of mouse peritoneal macrophages with sulfhydryl agents or H_2O_2 induces the activity of the cysteine transport across plasma membrane. The increase in cysteine transport into the cell enhances the synthesis of GSH, which protects the cell against these insulting agents[24]. GSH is one of the protective mechanisms that serve to limit the oxidative damage. Determining GSSG/GSH ratio is an accurate, specific and sensitive way of evaluating oxidative stress[25]. In the present study, GSH levels were reduced and GSSG levels increased by 7β-OH supplementation in a dose-dependent manner at all intervals. On comparison, no significant change was seen in GSSG levels of cholesterol exposed cells, while GSH levels declined at 7.5 μg/ml concentration at longer incubation intervals of 18 and 24 hr.

Earlier[26], it was reported that decrease in the intracellular levels of GSH by L-buthionine-(S,R)-sulfoximine (BSO), a selective inhibitor of γ-glutamyl-cysteine synthase enhanced the susceptibility of endothelial cells to Ox-LDL toxicity. By increase in intracellular levels of GSH, by L-2-oxothiazolidine-4-carboxylate (OTC), a promoter of GSH synthesis, the susceptibility of Ox-LDL toxicity was also reduced. Thus, the toxicity by Ox-LDL is dependent on the levels of intracellular GSH. Cell death due to cytotoxicity leads to formation of advanced plaques and hence depletion of GSH due to cholesterol and in particular 7β-OH might be responsible for the progression of atherosclerotic

Table 1—Effect of cholesterol and 7-β-hydroxy cholesterol (7-β-OH) on intracellular oxidized glutathione (GSSG) and reduced glutathione (GSH) levels and redox ratio (GSSG/GSH) levels in the cell lysates of murine peritoneal macrophages cultured at different incubation intervals

[Values are mean ± SEM of quadruplicates].

	Incubation intervals (hr)			
	6	12	18	24
GSSG levels (nmoles/mg protein)				
Cholesterol (μg/ml)				
0	5.25±0.25	5.25 ± 0.25	5.32 ± 0.25	5.4 ± 0.31
2.5	5.25±0.25	5.25 ± 0.26	5.4 ± 0.26	5.5 ± 0.28
5	5.35±0.25	5.35 ± 0.25	5.55 ± 0.25	5.8 ± 0.25
7.5	5.55±0.25	5.5 ± 0.35	5.7 ± 0.24	6.1 ± 0.31
7β-OH (μg /ml)				
0	6.32±0.26	6.5±0.25	6.7 ± 0.3^{a}	6.7 ± 0.32
2.5	6.32 ± 0.26	6.8 ± 0.1^{a}	6.4 ± 0.32	9.0 ± 0.26$^{\#\#aa}$*
5	6.95 ± 0.21^{a}	7.65 ± 0.3^{aa}	9.1 ± 0.26$^{\#\#aa}$**	12.1 ± 0.26$^{\#\#\#aaa}$***
7.5	7.75 ± 0.32^{a}*	9.1 ± 0.19$^{\# aa}$**	9.92 ± 0.29$^{\#aa}$**	13.3 ± 0.32$^{\#\#\#aaa}$***
GSH Levels (nmoles/mg protein)				
Cholesterol (μg/ml)				
0	70.75 ± 5.9	70.75 ± 3.1	72.25 ± 2.5	71.1 ± 3.7
2.5	71.50 ± 5.6	69.0 ± 3	68.5 ± 3.6	65.3 ± 2.6
5	71.75 ± 7.5	67.0 ± 2.7	65.6 ± 4.5	60.5 ± 2.4
7.5	71.5 ± 6.9	64.0 ± 2.8	62.2 ± 1.7*	55.7 ± 2.1*
7β-OH (μg /ml)				
0	77.7 ± 3.8	75.2 ± 4.0	69.7 ± 3.65	67.0 ± 4.0
2.5	61.5 ± 3.4*	58.5 ± 3.25	55.3 ± 3.25*	50.2 ± 3.25*^{a}
5	55.0 ± 2.9 *	52.2 ± 3.0	43.7 ± 2.5 $^{\#}$*^{a}	38.1 ± 5.5*^{a}
7.5	48.5 ± 2.5 *^{a}	43.1 ± 4.7 *^{a}	31.5 ± 2.2 $^{\#}$**^{aa}	28.5 ± 2.5**$^{aa\#\#}$
Redox Ratio (GSSG/GSH)				
Cholesterol (μg/ml)				
0	0.07 ± 0.006	0.07 ± 0.007	0.07± 0.005	0.07 ± 0.005
2.5	0.07 ± 0.008	0.07 ± 0.007	0.07 ± 0.005	0.08 ± 0.007
5	0.07 ±0.001	0.07 ± 0.001	0.08 ± 0.003$^{\#}$	0.09 ± 0.005$^{\#}$
7.5	0.07 ± 0.005	0.08 ± 0.007	0.09 ± 0.004$^{\#}$	0.1 ± 0.002$^{\#\#**}$
7β-OH (μg /ml)				
0	0.08± 0.007	0.08 ± 0.007	0.09 ± 0.002 ^{a}	0.10± 0.002^{a}
2.5	0.10± 0.006^{a}	0.11 ± 0.006^{a}	0.11 ± 0.007^{a}	0.17± 0.004$^{\#\#aa}$**
5	0.12± 0.001^{aaa}	0.14 ± 0.005$^{\#aaa}$	0.20 ± 0.003$^{\#\#\#aaa}$**	0.31± 0.002$^{\#\#\#aaa}$***
7.5	0.15± 0.005^{aaa}	0.21 ± 0.000^{aaa}	0.31 ± 0.003$^{\#\#\#aaa}$***	0.46±0.003$^{\#\#\#aaa}$***

*p< 0.05 ,**p< 0.01 and ***p<0.001 represent the comparison between control (untreated cells) and cells exposed to cholesterol/7β-OH for each incubation interval.

$^{\#}p$< 0.05 ,$^{\#\#}$ p< 0.01 and $^{\#\#\#}$ p< 0.001 represent the comparison between cells at incubation interval of 6 hr and other intervals for each concentration of cholesterol or 7β-OH tested.

^{a}p< 0.05,^{aa} p< 0.01 and ^{aaa}p<0.001 represent the comparison between the cells exposed to cholesterol and those exposed to 7-β-OH at the same concentration for each incubation interval

Table 2—Effect of cholesterol and 7-β-hydroxycholesterol on heat shock protein 70 (hsp70) production in murine peritoneal macrophages cultured at different incubation intervals
[Values are mean ± SEM of quadruplicates]

	Incubation intervals (hr)			
	6	12	18	24
Hsp70 levels ($A_{405\ nm}$)				
Cholesterol (μg/ml)				
0	0.056± 0.0005	0.058± 0.0010	0.059± 0.001	0.059 ± 0.001
2.5	0.053± 0.0005	0.057± 0.0004$^{\#}$	0.061± 0.0005$^{\#\#}$	0.061 ± 0.001$^{\#\#}$
5	0.053± 0.0004*	0.056± 0.0005$^{\#}$	0.058± 0.0010$^{\#}$	0.059 ±0.001$^{\#\#}$
7.5	0.052± 0.0005*	0.054± 0.0005*	0.055± 0.0005$^{\#*}$	0.054 ± 0.0005$^{\#\#*}$
7β-OH (μg/ml)				
0	0.056± 0.0005	0.058± 0.0005	0.059± 0.001	0.059± 0.002
2.5	0.057± 0.0005^{a}	0.056± 0.0005	0.057± 0.001^{a}	0.059± 0.001
5	0.053± 0.0005*	0.053± 0.0005^{a}**	0.052± 0.001^{a}**	0.050± 0.002^{aa}**
7.5	70.053± 0.001*	0.050± 0.0005^{aa**}	0.049± 0.001$^{\#aa**}$	0.046±0.001**$^{aa\ \#\#}$

*p<0.05 and **p < 0.01 represent the comparison between control (untreated cells) and the cells exposed to cholesterol/7β-OH for each incubation interval.
$^{\#}p$<0.05 and $^{\#\#}p$< 0.01 represent the comparison between cells at incubation interval of 6 hr and other intervals for each concentration of cholesterol or 7β-OH tested.
^{a}p< 0.05 and ^{aa}p< 0.01 represent the comparison between the cells exposed to cholesterol and 7β-OH at the same concentration for each incubation interval.

lesion. As 7β-OH cause significant decline in GSH levels, compared to cholesterol indicating that it is responsible for severe oxidative stress. The decline in GSH levels was also reported earlier in coronary venucular endothelial cells exposed to oxidative stress produced by xanthine oxidase and hypoxanthine[27].

Further, in the present study, at each interval, cholesterol showed a significant decrease in hsp70 level at 7.5 μg/ml concentration, while 7β-OH showed a significant decrease even at 5 μg/ml. At longer incubation interval also compared to cholesterol, 7β-OH significantly decreased hsp70 levels at 5 and 7.5 μg/ml indicating that 7β-OH may be responsible for reducing the hsp70 expression, as a result of which the cytoprotective effects of hsp70 are diminished. Earlier, it has been found that oxysterols from Ox-LDL are cytotoxic, but fail to induce hsp70 expression in endothelial cells[28].

Several reports have addressed the relation between the cellular levels of GSH and GSSG and modulation of hsp70 expression[29,30]. GSH and GSSG levels have significant effect on the stability and denaturation of cellular protein and depletion of intracellular glutathione could lead to oxidation of protein thiols, thereby trapping the proteins in an unstable three-dimensional conformation and hence initiating stress response[31]. Cellular glutathione depletion is also implicated in decreased total and specific heat-stress protein synthesis in various cell models[30,32] by impairing transcriptional activation of heat shock genes by inhibiting the nuclear translocation of Hsf-1[33].

It has been reported[34] that when cells are depleted of GSH to <5% of control, the relative amounts of specific hsps are also decreased. Both cholesterol and 7β-OH are found to deplete glutathione in a dose-dependant manner. The decrease in hsp70 can be considered as a result of it, since the maintenance of ratio reduced to oxidized glutathione is reported to be involved in the regulation of protein translation[35].

However, as hsp70 has been detected in atherosclerotic plaques[36], it is very likely that its high levels in the atherosclerotic lesions are induced by some other component of Ox-LDL, but not by oxysterols, such as 7β-OH and cholesterol. Thus, the inability of 7β-OH to activate the cells rescue mechanisms — hsp70 protein production and glutathione synthesis may lend support towards the creation of pro-atherogenic conditions.

References

1 Berliner J A & Heinecke J W (1996) *Free Radic Biol Med* 20, 707-727
2 Lemarie S, Lizard G, Monier S, Carole M, Gueldry S, Volvot F, Gambert P & Neel D (1998) *FEBS Lett* 440, 434-439

3 Hansson G K, Holm J & Jonasson L (1989) *Am J Pathol* 135, 169-175

4 Stemme S, Faber B, Holm J, Wiklund O, Witzum J L & Hansson G K (1995) *Proc Natl Acad Sci* 92, 3893-3897

5 Hessler J R, Robertson Jr. A L & Chisolm G M (1979) *Atherosclerosis* 32, 213-229

6 Hessler J R, Morel D W, Lewis L J & Chisolm G M (1983) *Arterioscler Thromb Vasc Biol* 3, 215-222

7 Deckert V, Persegoi L, Veins L, Lizard G, Athias A, Lallemant C, Gambert P & Lagrost L (1997) *Circulation* 95, 723-731

8 Reed D J (1986) *Biochem Pharmacol* 35, 7-13

9 Kosower N S & Kosower E M (1978) *Int Rev Cytol* 54, 109-160

10 Kidd P M (1997) *Alt Med Rev* 2,155-176

11 Nathan C F, Silverstein S C, Brukner L H & Cohn Z A (1979) *J Exp Med* 149, 100 -113.

12 Nathan C F (1980) in *Mononuclear Phagocytes, Functional Aspects* (van Furth R, ed.), pt. II, pp. 1165-1186, Martinus Nijhoff, The Hague

13 Meister A & Anderson M E (1983) *Annu Rev Biochem* 52, 711-760

14 Pichoner H, Metdiewa D & Winterbourn C C (1995) *Arch Biochem Biophys* 323, 429-437

15 Welch W J (1990) in *Stress Proteins in Biology and Medicine* (Morimoto R I, Tissieres A & Geogopoulos C, eds.), pp. 223-278, Cold Spring Harbor Laboratory Press, NY

16 Parsell D A & Lindquist S (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones*, pp 457-494. Cold Spring Harbor Laboratory Press, NY

17 Chong K Y, Lai C C, Lille S, Chang C & Su C Y (1998) *J Mol Cell Cardiol* 30, 599-608

18 Simon M M, Reikerstorfer A, Schwarz A, Krone C, Luger T A, Jaatela M & Schwartz T (1995) *J Clin Invest* 95, 926-933

19 Johnson DD, Berbarian P A & Bond M G (1990) *Atherosclerosis* 84, 114-119

20 Bansal G, Singh U & Bansal M P (2001) *Cell Physiol Biochem* 11, 241-246

21 Lizard G, Monier S, Cordelet C, Gesquiere L, Deckert V, Gueldry S, Lagrost L & Gambert P (1999) *Arterioscler Thromb Vasc Biol* 19,1190-1200

22 Hissin P J & Hilf R (1976) *Anal Biochem* 74, 214-226

23 Lowry O H, Rosebrough N J, Farr A L & Randall R J (1951) *J Biol Chem* 193, 265-275

24 Bannai S, Sato H, Ishii T & Taketani S (1991) *Biochem Biophys Acta* 1092, 175-179

25 Villa-Caballero L, Nava-Ocampo A A, Frati-Munari A C & Ponse Monter H (2000) *Gac Med Mex* 136, 249-256

26 Kuzuya M, Naito M, Funaki C, Hayashi T, Asai K & Kuzuya F (1989) *Biochem Biophy Res Commun* 163, 1466-1472

27 Aucoin M M, Barhoumi R, Kochevar D T, Granger H T & Burghartt R C (1995) *Am J Physiol* 206 (*Heart Circ Physiol*, 37), 41651-41658

28 Pirillo A, Zhu W, Roma P, Galli G, Caruso D, Pellagatta F & Catapano A L (1999) *FEBS Lett* 462,113-116

29 Lord Fontaine S & Averill-Bates D A (2002) *Free Radic Biol Med* 32,752-765

30 Huang L E, Zhang H, Bae S W & Liu A Y-C (1994) *J Biol Chem* 269, 30718-30725

31 Freeman M L, Huntley S A, Meredith M J, Senisterra G A & Lepock J (1997) *Cell Stress Chaperones* 2, 191-198

32 Mitchell J B & Russo A (1983) *Rad Res* 95, 471-485

33 Rokutan K, Hirakawa T, Teshima S, Honda S & Kishi K (1996) *J Clin Invest* 97, 2242-2250

34 Russo A, Mitchell J B & McPherson S (1984) *Br J Cancer* 49, 753-758

35 Kosower N S, Vanderhoff G A & Kosower E M (1972) *Biochim Biophy Acta* 272, 623-637

36 Berbarian J A, Meyers W, Tytell M, Challa V & Bond M G (1990) *Am J Physiol* 136, 71-80