Macrophage populations of different origins have distinct susceptibilities to lipid peroxidation induced by β-haematin (malaria pigment)

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Abstract **We investigated the susceptibility of peritoneal mouse macrophages and macrophage and microglia cell lines to the peroxidative activity of β-haematin, the synthetic polymer identical to native malaria pigment. The extent of lipid peroxidation, measured as production of thiobarbituric acid reactive substances (TBARS), was greater for peritoneal macrophages than for cell lines and microglia cells. TBARS production apparently was not attributable to the release of free iron from the protoporphyrin moiety, but related to lower glutathione content and different lipid composition of the cell membrane. These findings offer a new interpretation for the contentious immunomodulatory effects of β-haematin reported for phagocytes of different origins.**

Key words: Macrophage; β-Hematin; Malaria pigment; Lipid peroxidation

1. Introduction

Phagocytes (e.g. monocytes-macrophages, neutrophils, etc.) of malaria-infected patients contain granules of pigment known as haemozoin (malaria pigment), as a result of the ingestion of intraerythrocytic malaria parasites, residual bodies (the remnant of a parasite after schizont rupture) or possibly free haemozoin [1–3]. Haemozoin – a polymer of haem units (ferriprotoporphyrin IX, Fe(III)-PPIX) linked through an iron carboxylate bridge [1] – appears to contribute to the production of inflammatory cytokines, such as TNFα or IL6, during acute malaria attacks [4–6]. Accumulation of pigment inside macrophages has also been shown to impair macrophage activation and functions. Repeated cycles of phagocytosis as well as the production of oxygen radicals and nitrogen oxides are inhibited [7,8]. In addition, we also showed that peritoneal mouse macrophages fed with β-haematin, a synthetic polymer of Fe(III)-PPIX identical to native haemozoin [9,10] failed to produce TNFα and Nitric Oxide (NO) when stimulated by lipopolysaccharide (LPS) [11]. Two aspects of this phenomenon are worth noting: (i) by contrast to peritoneal macrophages, microglia cells derived from the same mouse strain proved quite refractory to the β-haematin-induced functional inhibition; (ii) the inhibition of NO and TNFα production observed in peritoneal macrophages was partially counterbalanced by the addition of sulphydryl group donors such as 2-mercaptoethanol, *N*-acetylcysteine and glutathione [11]. These findings suggest the involvement of oxidative mechanisms in the intracellular activity of β-haematin, due to the known properties of haem-containing molecules as catalysts of lipid peroxidation [12]. This prompted us to verify whether (i) β-haematin has prooxidant activity in macrophages and (ii) the ability of the different macrophage populations to cope with the β-haematin-induced oxidative stress is related to a different membrane lipid composition and/or to the level of antioxidant defences.

2. Materials and methods

2.1. Mice

Specific pathogen-free female CD1 mice, 6–8 weeks old, were obtained from Charles River Italia (Calco, Como, Italy). Mice were maintained under conventional conditions and fed standard mouse pellet and water ad libitum.

2.2. Culture medium and reagents

The medium used (referred to as complete medium, CM) was RPMI 1640 (HyClone Europe Ltd., Cramlington, NL) supplemented with 100 IU/ml of penicillin, 2 mM glutamine, 100 μg/ml streptomycin, 20 mM HEPES buffer and 5% FCS (HyClone Europe). Reagents such as haematin (ferriprotoporphyrin IX-hydroxide, H-3505), reduced glutathione (GSH, G 4251), picryl sulphonic acid, were purchased from Sigma-Italia (Milan, Italy); thiobarbituric acid, 5′,5′-dithiobis-(2-dinitro) benzoic acid (DTNB) and NADPH from Fluka (Buchs, Switzerland) and glutathione reductase from Boehringer Mannheim (Germany). Haematin was freshly dissolved in NaOH 0.1 M and diluted to the appropriate concentration with CM immediately before use (final pH 7.4). β-Haematin was synthesised as previously reported [9,13] from a solution of haematin precipitated by the addition of acetic acid. After overnight incubation at 70°C, the precipitate was washed four times with distilled water. Unreacted haematin was removed by extracting the precipitate twice for 3 h in 0.1 M sodium bicarbonate buffer at pH 9.1. The purity of the final product was routinely controlled by infrared spectroscopy [9]. Batches of β-haematin, which did not meet standard criteria for purity, were discarded. To facilitate the treatment of macrophage monolayers with insoluble β-haematin, the compound was resuspended in CM and mechanically microdispersed by sonication.

2.3. Cells

The cells used were: proteose-peptone elicited peritoneal macrophages from CD1 mice; J774.1 macrophage cell line and BV2, a retrovirus transformed microglia cell line of C3H/HeJ origin [14]. Cells were maintained in CM and routinely split. For the lipoperoxidation experiments, cells were seeded at $5–6\times10^6$/ml and treated for 4 h with β-haematin or soluble haematin (50 μg/ml) or cultured in CM only. Cell viability was assessed by trypan blue exclusion and metabolic activity [11]. Cell homogenate was prepared by subjecting cell suspension to two cycles of freezing-thawing followed by a brief sonication and total protein content was determined according to Smith [15]. The amount of phagocytised β-haematin was determined by taking an aliquot of the cell homogenate, solubilising it in 1 N NaOH and reading the absorbance of the clear supernatant at 385 nm.

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2.4. Lipid peroxidation experiments

Endogenous and iron-induced lipid peroxidation was measured by determining the levels of thiobarbituric acid reactive substances (TBARS) in cell homogenate [16]. Briefly, total cell homogenate (100–200 μg protein) was incubated at 37°C for 60 min in the presence of 100 μM $FeSO_4$ in 1 mM Tris-HCl, 154 mM NaCl, 0.1 mM EDTA, pH 7.4. Aqueous solution of $FeSO_4$ was prepared immediately before use. Endogenous TBARS formation was measured in the absence of $FeSO_4$. TBARS were quantified using 1,1,3,3-tetraethoxypropane for the calibration curve and the results expressed as nmol/mg protein. To avoid overestimation of TBARS production due to the interference of haem compounds in cells containing haematin or β-haematin, adjustment for absorbance at 532 nm was made in accordance to Gilbert et al. [17]. The conditions of incubation were determined in pilot experiments showing that TBARS formation was essentially complete by 45 min and reached a plateau at 100 μM $FeSO_4$.

2.5. Total glutathione and glutathione reductase (GR) determination

For total glutathione (GSH+GSSG) determination, an aliquot of the cell homogenate was precipitated, immediately after preparation, with 1% (w/v) picric acid. Total glutathione was spectrophotometrically determined in the supernatant in the presence of GR, NADPH and DTNB [18]. GR activity was determined in accordance with Pinto et al. [19] on the clear supernatant obtained by centrifugation of the total homogenate at 5500×*g* for 40 min.

2.6. Total lipid extraction and analysis

The cell membrane fraction was obtained by centrifugation of the total homogenate at 5500×*g* for 40 min. Total lipids were extracted in accordance with Folch et al. [20] and aliquots of the lipid extract were taken for the phospholipid phosphorus [21] and total cholesterol determination (Boehringer-Mannheim kit). Total fatty acid composition was determined by gas liquid chromatography [22]. The degree of unsaturation of fatty acids was calculated and expressed as double bond index (DBI). DBI represents the sum of the values obtained by multiplying the percentage of each fatty acid by the number of double bonds in that acid, divided by 100.

2.7. Statistics

Data are reported as mean ± standard deviation of the experiments conducted. Results were compared by using the analysis of variance (ANOVA).

3. Results and discussion

Peritoneal exudate macrophages, J774.1 macrophage like cells and BV2 microglial cells proved able to readily ingest β-haematin: intracellular pigment could be detected at the end of the 4-h treatment in 75–80% of the cells, as confirmed by light and electron microscopy [11]. The extent of phagocytosis was linearly dependent upon the concentration of β-haematin and corresponded approximately to 30–50% of the initial amount of pigment, with no significant differences among the different cell types.

After exposure to β-haematin, and even more so with unpolymerised haematin, TBARS production by peritoneal macrophages was higher than that of BV2 or J774 cells (Fig. 1). The possibility that the β-haematin-induced TBARS production could be due to the release of iron from the porphyrin molecule was indirectly excluded because the extent of TBARS production was not reduced in cells treated with β-haematin in the presence of 150 μM desferroxamine, a potent iron chelator and inhibitor of iron-dependent lipid peroxidation [23] (data not illustrated here). Similarly, β-haematin-induced TBARS production was not increased: (i) when cell homogenates were incubated at pH 5.5 (condition simulating the internal acidic environment of phagolysosome which could favour the release of free iron from the protoporphyrin molecule) [7]; (ii) in the presence of 0.1 mM hydrogen peroxide (ruling out the involvement of hydroxyl radicals generated through an Haber-Weiss reaction) [24].

Peritoneal macrophages appeared to be inherently more susceptible than BV2 and J774 cells to oxidative stress as they showed comparatively higher levels of endogenous as well as iron-induced TBARS production, either in the presence or in the absence of the pigment (Fig. 1). The possibility that different cell proliferative activities could be the cause of the observed differences in sensitivities was dismissed because both unmodified and growth-arrested (by overnight treatment with 100 U/ml of interferon-γ) BV2 cells produced similar amounts of endogenous or β-haematin evoked TBARS (not shown).

Membrane fatty acid composition and fluidity are amongst the factors known to influence the cell susceptibility to lipid peroxidation. Lipid peroxidation is associated with a variety of pathological events [25,26]. Impairment of cell functions is ascribed to changes in the bilayer properties, such as a reduction of membrane fluidity and inactivation of membrane-bound enzymes [27–29]. The membranes of microglia and J774 cells are rich in oleic acid (C18:1) and low in linoleic (C18:2 n-6) and arachidonic acid (C20:4 n-6) (Table 1). This pattern is similar to that reported for other types of tumour cells [30], but significantly different from that of peritoneal

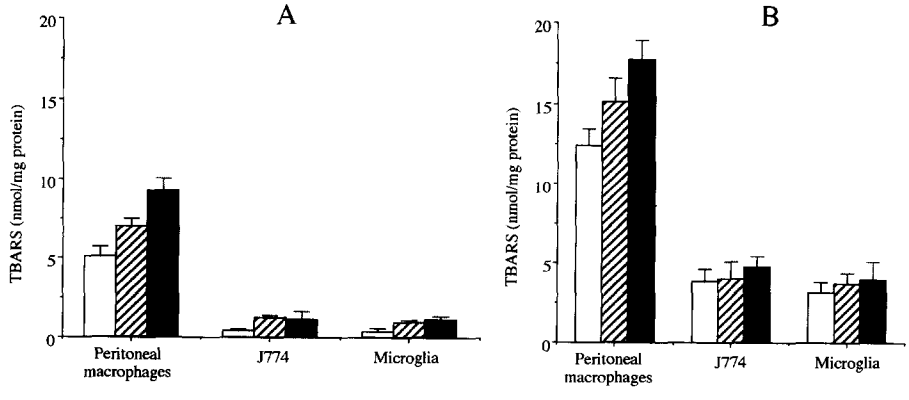

Fig. 1. Increased TBARS production in different macrophage populations after treatment with 50 μg/ml of β-haematin (hatched bars) or 50 μg/ml of soluble haematin (solid bars) compared to control cells (empty bars). A: Endogenous TBARS production (peritoneal macrophages significantly different from J774 and microglia cells, $n = 10$, $P < 0.01$). B: Iron-induced TBARS production (100 μM $FeSO_4$, 60 min, 37°C) (peritoneal macrophages significantly different from J774 and microglia cells, $n = 10$, $P < 0.01$).

Table 1
Molar percent of lipid fatty acids in control and β-haematin-treated macrophages[a]

Fatty acids	Peritoneal macrophages	Peritoneal macrophages +β-haematin	J774	J774 +β-haematin	Microglia cells	Microglia cells +β-haematin
C16:0	35.8 ± 2.2	39.5 ± 1.5	35.6 ± 3.1	34.3 ± 1.2	32.1 ± 0.5	28.9 ± 0.3
C16:1	3.3 ± 0.5[b]	5.1 ± 1.3	8.8 ± 0.5	9.1 ± 0.8	12.8 ± 1.6	13.5 ± 0.7
C18:0	22.8 ± 1.2[b]	21.6 ± 0.8	10.1 ± 0.5	9.4 ± 0.1	9.8 ± 2.3	8.4 ± 1.2
C18:1	14.7 ± 0.1[b]	14.0 ± 1.0	34.0 ± 0.2	36.9 ± 0.1	38.1 ± 3.0	43.6 ± 0.9
C18:2 n-6	10.0 ± 1.0[c]	8.0 ± 0.8	3.5 ± 0.1	3.3 ± 0.7	2.0 ± 0.5	1.8 ± 0.2
C20:4 n-6	11.0 ± 0.4[b]	9.0 ± 0.7[d]	5.8 ± 0.8	5.2 ± 1.5	3.2 ± 0.1	2.8 ± 0.1
C22:6 n-3	2.1 ± 0.4	2.0 ± 0.7	2.2 ± 0.6	1.8 ± 0.5	1.3 ± 0.2	1.0 ± 0.1
DBI[e]	0.95		0.86		0.76	
S/U[f]	1.4		0.84		0.73	

[a]Data represent the mean ± standard deviation ($n = 4$).
[b]Statistically different from J774 and microglia cells ($P < 0.01$ by ANOVA test).
[c]Statistically different from J774 and microglia cells ($P < 0.02$ by ANOVA test).
[d]Statistically different from untreated peritoneal macrophages ($P < 0.01$ by ANOVA test).
[e]DBI represents the sum of the values obtained by multiplying the percentage of each fatty acid by the number of double bonds in that acid, divided by 100.
[f]S/U = saturated/unsaturated fatty acid ratio.

macrophages, that are richer in C18:2 n-6 and C20:4 n-6 PUFA. Fatty acid composition of peritoneal macrophage accounts for the higher DBI compared to microglia and J774 cells. This latter finding is in agreement with other reports [31] and might favour the production of large quantities of C20:4-derived eicosanoids in macrophages [32]. Significantly, all the three cell populations have low contents of docosahexaenoic acid (C22:6 n-3), one of the main substrates for membrane lipid peroxidation.

In peritoneal macrophages, the high proportion of PUFA is compensated for by a lower content of monounsaturated species, palmitoleic (C16:1) and oleic (C18:1) fatty acids, and by an increase in stearic acid (C18:0), leading to a higher saturated/unsaturated (S/U) fatty acid ratio.

The analysis of the cell fatty acid pattern after 4-h treatment with β-haematin confirms that the polymer induces peroxidative stress, which coincides with a significative loss of C20:4 n-6 and a smaller decrease of C18:2 n-6 fatty acids in peritoneal macrophages, but not in microglia cells (Table 1). This finding is in agreement with data showing that native haemozoin and β-haematin oxidise arachidonic acid to hydroxyeicosatetraenoic acids [33].

The total phospholipid content, however, was significantly lower in peritoneal macrophages than microglia and J774 cells (Table 2). This suggests that other factors, such as membrane fluidity and/or antioxidant defences (additional to the higher content in PUFA), contribute to the greater susceptibility to peroxidation of peritoneal macrophages.

Microglia, J774 and peritoneal macrophages were thus analysed for total cholesterol content. Despite a lower cholesterol content, the cholesterol/phospholipid molar ratio was approximately 50% higher in peritoneal macrophages compared to microglia and J774 cells (Table 2). This, along with the higher S/U fatty acid ratio, suggests a lower degree of membrane fluidity in peritoneal macrophages, possibly associated with increased lipid peroxidation [34,35]. Lipid peroxidation might be further enhanced by the fact that haematin (and possibly β-haematin too) would interact more strongly with more tightly packed bilayers [36,37].

The antioxidant defence system of the different macrophage populations was assessed by measuring the level of total glutathione and the activity of glutathione reductase (GR), a NADPH- dependent enzyme implicated in maintaining GSH/GSSG homeostasis. GSH and GR play a particularly important role in protecting macrophages from oxidative stress during phagocytosis, when a number of reactive oxygen intermediates are generated [38,39]. Table 3 shows that total glutathione content was significantly lower in peritoneal macrophages than in microglia and J774 cells, while GR activity did not vary significantly among the three cell populations. The low GSH content, reducing the peritoneal macrophages' capability to cope with the oxidative stress induced by β-hae-

Table 2
Protein, cholesterol (CHO) and phospholipid (PL) content and CHO/PL molar ratio in different macrophage populations

Cell type	Proteins (μg/10^6 cells)	Cholesterol (nmol/10^6 cells)	Phospholipids (nmol/10^6 cells)	CHO/PL
Peritoneal macrophages	40 ± 5.0[a]	2.2 ± 0.1[a]	7.3 ± 0.4[a]	0.301
J774	182 ± 15.0	5.6 ± 0.9	28.5 ± 3.2	0.196
Microglia cells	193 ± 12.0	7.4 ± 0.5	33.8 ± 2.0	0.219

[a]Statistically different from J774 and microglia cells ($P < 0.01$ by ANOVA test, $n = 6$).

Table 3
Total glutathione content and GSH reductase activity of different macrophage populations

Cells	Glutathione (nmol/mg protein)	GSH reductase (mU/mg protein)
Peritoneal macrophages	27.5 ± 3[a]	67.2 ± 4
J 774	42.6 ± 2	69.1 ± 4
Microglia cells	56.3 ± 5	57.1 ± 2

[a]Statistically different from J774 and microglia cells ($P < 0.01$ by ANOVA test, $n = 4$).

matin, can further increase cell susceptibility to peroxidative damage.

4. Conclusions

The present study shows that unpolymerised and polymerised Fe(III) protoporphyrin IX (synthetic β-haematin, identical to trophozoite-isolated pigment) exert a prooxidant activity, apparently not due to the release of free iron. Susceptibility to such peroxidative stress varies with the macrophage cell type and is related to the membrane composition and the cell antioxidant defences. The higher susceptibility of peritoneal macrophages compared to microglial cells and macrophage cell lines can be ascribed to their higher content in PUFA and lower membrane fluidity and GSH levels. In addition, we have preliminary data indicating that restoring GSH levels by treatment of β-haematin-fed peritoneal macrophages with GSH results in a partial reduction of TBARS production.

We had already reported that treatment of peritoneal macrophages with β-haematin results in impaired macrophage functions, and that the inhibition of NO and TNFα production by β-haematin-treated peritoneal macrophages is partially counterbalanced by the addition of sulphydryl group donors or glutathione [11].

The current study allows us to postulate that the impairment of the biological functions upon exposure to β-haematin is related to a peroxidative stress to which peritoneal macrophages are eminently susceptible. It is also likely that the decrease in unsaturated fatty acids caused by the prooxidant activity of β-haematin impairs other key macrophage functions, such as adhesion and phagocytosis [40].

These findings shed new light on the controversial issue of the immunomodulatory effects of β-haematin reported for phagocytes obtained from different species and tissues. They also imply that phagocytes residing in different anatomical districts and characterised by different membrane composition and antioxidant defences would respond differently after ingesting the malaria pigment. The implications and relevance of these results to the pathophysiology of malaria will need to be further investigated.

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References

[1] Francis, S.E., Sullivan, D.J. and Goldberg, D.E. (1997) Annu. Rev. Microbiol. 51, 97–123.
[2] Nguyen, P.H., Day, N., Pram, T.D., Ferguson, D.J. and White, N.J. (1995) Trans. R. Soc. Trop. Med. Hyg. 89, 200–204.
[3] Metzger, W.G., Mordmuller, B.G. and Kremsner, P.G. (1995) Trans. R. Soc. Trop. Med. Hyg. 89, 637–638.
[4] Pichyangkul, S., Saengkrai, P. and Webster, H. (1994) Am. J. Trop. Med. Hyg. 51, 430–435.
[5] Prada, J., Malinowsky, J., Muller, S., Bienzle, U. and Kremsner, P. (1995) Eur. Cytokine Netw. 6, 109–112.
[6] Sherry, B., Alava, G., Tracey, K., Martiney, J., Cerami, A. and Slater, A. (1995) J. Inflamm. 45, 85–96.
[7] Arese, P. and Schwarzer, E. (1997) Ann. Trop. Med. Parasitol. 91, 501–516.
[8] Prada, J., Malinoski, J., Muller, S., Bienzle, U. and Kremsner, P. (1996) Am. J. Trop. Med. Hyg. 54, 620–662.
[9] Basilico, N., Monti, D., Olliaro, P. and Taramelli, D. (1997) FEBS Lett. 409, 297–299.
[10] Bohle, S., Dinnebier, R.E., Madsen, S.K. and Stephens, P.W. (1997) J. Biol. Chem. 272, 713–716.
[11] Taramelli, D., Basilico, N., Pagani, E., Grande, R., Monti, D., Ghione, M. and Olliaro, P. (1995) Exp. Parasitol. 81, 501–511.
[12] Tappel, A. (1955) J. Biol. Chem. 217, 721–733.
[13] Egan, T., Ross, D. and Adams, P. (1994) FEBS Lett. 352, 54–57.
[14] Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R. and Bistoni, F. (1990) J. Neuroimmunol. 27, 229–237.
[15] Smith, P., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. Biochem. 150, 76–85.
[16] Buege, J.A. and Aust, S.D. (1978) in: Methods in Enzymology (Fletcher, S. and Packer, S., Eds.), Microsomal Lipid Peroxidation, Vol. 52, pp. 302–310, Academic Press, New York.
[17] Gilbert, H., Stump, D. and Roth, E. (1984) Anal. Biochem. 137, 282–286.
[18] Griffith, O.W. (1985) in: Methods of Enzymatic Analysis (Bergmeyer, H.V., Ed.), Glutathione and Glutathione Disulphide, Vol. VIII, pp. 521–529, Academic Press, New York.
[19] Pinto, M.C., Mata, A.M. and Lopez-Barea, J. (1984) Arch. Biochem. Biophys. 228, 1–12.
[20] Folch, J., Lees, M. and Sloane Stanley, G.M. (1957) J. Biol. Chem. 226, 497–509.
[21] Bartlett, G. (1959) J. Biol. Chem. 234, 466–468.
[22] Rizzo, A.M., Galli, C.F., Montorfano, G. and Berra, B. (1995) J. Neurochem. 64, 1728–1733.
[23] Gutteridge, J.M., Richmond, R. and Halliwell, B. (1979) Biochem. J. 184, 469–472.
[24] Gutteridge, J.M. and Halliwell, B. (1989) Clin. Hematol. 2, 195–256.
[25] Ames, B.N. (1983) Science 221, 1256–1264.
[26] Cerutti, P. (1985) Science 227, 375–381.
[27] Rice-Evans, C. and Hochstein, P. (1981) Biochem. Biophys. Res. Commun. 100, 1537–1542.
[28] Kukreja, R., Okabe, E., Schrier, G.M. and Hess, M.L. (1988) Arch. Biochem. Biophys. 261, 4447–4457.
[29] Ohta, A., Mohri, T. and Ohyashiki, T. (1989) Biochim. Biophys. Acta 984, 151–157.
[30] Calorini, L., Fallani, A., Tombaccini, D., Barletta, E., Mugnai, G., Di Renzo, M.F., Comoglio, M.P. and Ruggieri, S. (1989) Lipids 24, 685–690.
[31] Scott, W.A., Zrite, J.M., Hamill, A.L., Kempe, J. and Cohn, Z.A. (1980) J. Exp. Med. 152, 324–335.
[32] Nathan, C.F. and Cohn, Z.A. (1985) in: Textbook of Rheumatology (Kelly, W.W., Harris, E.D., Ruddy, S. and Sledge, C.B., Eds.), pp. 144–169, W.B. Saunders, Philadelphia, PA.
[33] Green, M., Xiao, L. and Lal, A. (1996) Mol. Biochem. Parasitol. 83, 183–188.
[34] McLean, L.R. and Hagaman, K.A. (1992) Free Radical Biol. Med. 12, 113–119.
[35] Cervato, G., Viani, P., Masserini, M., Di Iorio, C. and Cestaro, B. (1988) Chem. Phys. Lipids 49, 135–139.
[36] Cannon, J., Kuo, F., Pasternack, R., Wong, N. and Muller-Heberhard, U. (1984) Biochemistry 23, 3715–3721.
[37] Schmitt, T.H., Frezzatti, W.A. and Schreier, S. (1993) Arch. Biochem. Biophys. 307, 96–103.
[38] Babior, B. (1984) Blood 64, 959–966.
[39] Chaudhry, A., Santinga, J. and Gabig, T.G. (1982) Blood 60, 979–983.
[40] Calder, P.C., Bond, J.A., Harvey, D.J., Gordon, S. and Newsholme, E. (1990) Biochem. J. 269, 807–814.