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Magnesium deficiency in vitro enhances free radical-induced intracellular oxidation and cytotoxicity in endothelial cells

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The effect of magnesium (Mg)-deficient culture on endothelial cell susceptibility to oxidative stress was examined. Bovine endothelial cells were cultured in either control sufficient (0.8 mM) or deficient (0.4 mM) levels of $MgCl_2$. Oxygen radicals were produced extracellularly by the addition of dihydroxyfumarate and Fe^{3+}-ADP. Isolated Mg-deficient endothelial cells produced 2- to 3-fold higher levels of thiobarbituric acid (TBA)-reactive materials when incubated with this free radical system. Additional studies were performed using digitized video microscopy and 2',7'-dichlorofluorescin diacetate (DCFDA) as an intracellular indicator for oxidative events at the single cell level. In response to the exogenous oxidative stress, endothelial cells exhibited a time-dependent increase in fluorescence, suggestive of intracellular lipid peroxidation. The increase in cellular fluorescence began within 1 min of free radical addition; the Mg-deficient cells exhibited a more rapid increase in fluorescence than that of Mg-sufficient cells. In separate experiments, cellular viability was assessed using the Trypan blue exclusion assay. Mg deficiency increased cytotoxicity of the added oxyradicals, but the loss of cellular viability began to occur only after 15 min of free radical exposure, lagging behind the detection of intracellular oxidation products. These results suggest that increased oxidative endothelial cell injury may contribute to vascular injury during Mg deficiency.

Magnesium deficiency; Free radical; Endothelial cell; Cell culture

1. INTRODUCTION

The molecular mechanisms of magnesium (Mg) deficiency-associated cardiovascular injury are poorly understood. Recent findings from our laboratory have implicated a role for increased oxidative stress during the Mg deficiency-induced tissue injury [1–5]. During Mg deficiency, serum 'non-protein-bound' iron increased 50% [6]; in addition, lipid peroxidation products, assayed as thiobarbituric acid (TBA)-reactive materials, increased 2-fold compared to control animals [7]. Our laboratory has also documented that Mg deficiency results in significant elevation of circulating inflammatory cytokines [8]. The endothelial cells may represent an initial cellular site of increased oxidative injury. We have recently focused our studies on free radical injury in endothelial cells cultured in normal Mg levels [9–11]. In the present communication, the effect of Mg-deficient culture media on the cellular response to an iron-catalyzed hydroxyl radical generating system was studied. By using the Meridian Laser Cytometry system, lipid peroxidation at the single cell level can be monitored and quantified continuously.

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2. MATERIALS AND METHODS

2.1. *Chemicals and cell culture*

Chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless stated otherwise. Bovine aortic endothelial cells (AG 07684) were obtained from The Coriell Institute for Medical Research, Camden, NJ, and cultured in minimal essential medium (MEM) for suspension cultures (MediaTech, Washington DC); the medium was supplemented with 15% calf serum, L-glutamine (4 mM final), 1% MEM vitamins (MediaTech), 1% MEM non-essential amino acids, 0.1% trace elements #1 and #2, HEPES buffer, $CaCl_2$ and sufficient Mg to achieve a final concentration of 0.4 mM or 0.8 mM Mg. The concentration of Mg was confirmed by atomic absorption spectra.

Cells were grown in either 150 -m^2 culture flask (Corning, Wexford, PA), 24-well multiplates (Corning), or on circular microscopic coverslips. For studies of the TBA reactivity of cells to oxygen radicals, near confluent cultures were digested with 0.05% trypsin in 0.02% EDTA solution; the trypsinization of cultured cells was stopped by adding growth medium. The cells were pelleted and washed twice with the incubation buffer: 10 mM glucose, 1.2 mM $MgCl_2$, 125 mM NaCl and 10 mM potassium phosphate, pH 7.2. The TBA reactivity of the isolated cells to oxygen radicals was initiated with the addition of 1.7 mM DHF + FeADP (50 μM $FeCl_3$ chelated by 500 μM ADP). The TBA-reactive materials formed after 30 min at 37°C of incubation were determined and expressed as malondiadehyde equivalents, as described previously [12,13].

2.2. *Single cell lipid peroxidation*

Endothelial cells were grown on 22-mm coverslips. The coverslips were removed from the growth media, washed twice in buffer, and then labeled with 5 μM DCFDA for 15 min immediately prior to use. Cellular fluorescence was measured at 515 nm following excitation at 488 nm using the ACAS 570 Interactive Laser Cytometer (Meridian Instruments Inc., Okemos, MI).

2.3. *Endothelial cell viability*

Endothelial cell viability was measured by uptake of Trypan blue

(0.1%). For the attached cells which were grown in disposable multiplates, Trypan blue was added for 1 min, followed by the addition of buffered glutaraldehyde solution (final concentration 1.5%) to fix the cells for counting. Cell counting was performed by a 'blinded' researcher who was unaware of the level of Mg in the tissue culture medium.

3. RESULTS AND DISCUSSION

3.1. *Cell culture*

We attempted to grow endothelial cells over a wide range of Mg concentrations. In our laboratory, 0.4 mM was the lowest concentration of Mg in which endothelial cells would grow. This finding was markedly different from that reported earlier by Banai et al. [14], who found that endothelial cells grew well at both 0.04 mM and 0.8 mM Mg. This difference in the Mg requirement for endothelial cell growth could be related to a number of significant differences in the cellular models. In the earlier study, endothelial cells were grown in gelatin-coated plates; an initial 2 to 12 h incubation period was used in proliferation experiments in which the cells were incubated in the presence of Mg, and the endothelial cells were from the adrenal cortex. In our experiments, the cells were washed in Mg-free media prior to plating in media containing the desired final Mg concentration. While endothelial cells are easily trypsinized, we found that cells grown in low Mg were more resistant to trypsinization. Because of this resistance to trypsinization, we routinely trypsinized both Mg-sufficient and Mg-deficient cells for the same period of time, and then used mechanical methods to help harvest Mg-deficient cells.

3.2. *Free radical induced TBA reactivity*

The cultured endothelial cells were sensitive to free radical-induced cytotoxicity; in association, significant increases in TBA-reactant formation occurred [9,10,15,16]. In the present study, when endothelial cells cultured in Mg-sufficient (0.8 mM) or Mg-deficient (0.4 mM) media were exposed to the free radical system under identical conditions (Fig. 1), the Mg-deficient cells produced greater than 2-fold-higher levels of TBA-reactive materials ($P < 0.01$). In addition, cells cultured in low Mg showed a markedly higher percentage of dead cells following this treatment (95%) than for cells cultured in normal Mg (55%). These results suggest that Mg deficiency increases the sensitivity of endothelial cells to free radical peroxidative injury. We have previously shown that Mg deficiency in vivo results in a similar increase in erythrocyte sensitivity to oxidative stress [4].

3.3. *Measurement of intracellular peroxide formation*

While there was no significant difference between the initial viability of Mg-deficient and Mg-sufficient cells, the need to use mechanical force to harvest Mg-deficient cells suggested that these cells may have been injured during harvesting. In addition, the TBA assay is a relatively non-specific method for measuring cellular lipid peroxidation. With the availability of the ACAS Meridian Laser Cytometry System, we were able to evaluate the influence of Mg deficiency on cellular resistance to lipid peroxidation of attached cells. We used DCFA as an intracellular probe to monitor and quantify cellular lipid peroxidation in response to exogenously added hydroxyl free radicals. The probe, 2',7'-dichlorofluorescin diacetate, is taken up by endothelial cells and deacetylated intracellularly to the non-fluorescent 2,7-dichlorofluorescin. The 2,7-dichlorofluorescin reacts quantitatively with either oxidative species or peroxides and hydroperoxides to produce fluorescent 2,7-dichlorofluorescein within the cell.

Using this fluorescent probe and digitized video microscopy, we were able to show that the addition of exogenous free radicals caused a marked increase in intracellular oxidation of the 2,7-dichlorofluorescin. While the oxidation of the dichorofluorescin to dichlorofluorescein is relatively non-specific, it will react with any peroxides or hydroperoxides formed within the cell; in experiments involving addition of an extracellular free radical source, the increase in fluorescence is a clear indicator of intracellular oxidative species. Fig. 2 demonstrates the response from a paired experiment in

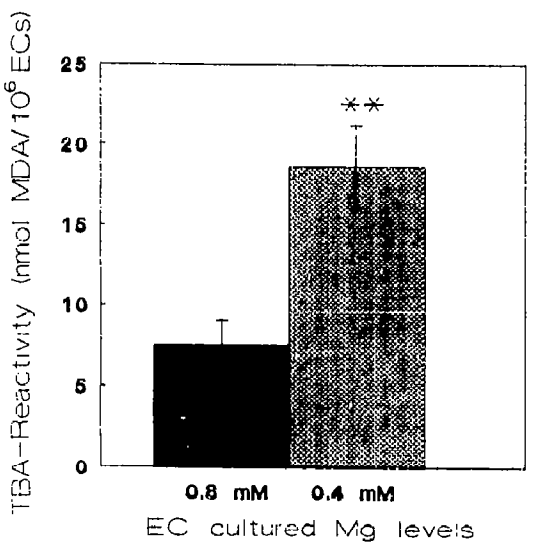

Fig. 1. Effect of cultured Mg levels on free radical-mediated TBA reactivity in isolated endothelial cells. Endothelial cells (10^6/ml) were incubated with the dihydroxyfumerate–FeADP free radical system for 30 min at 37°C. $^{**}P < 0.01$ between groups.

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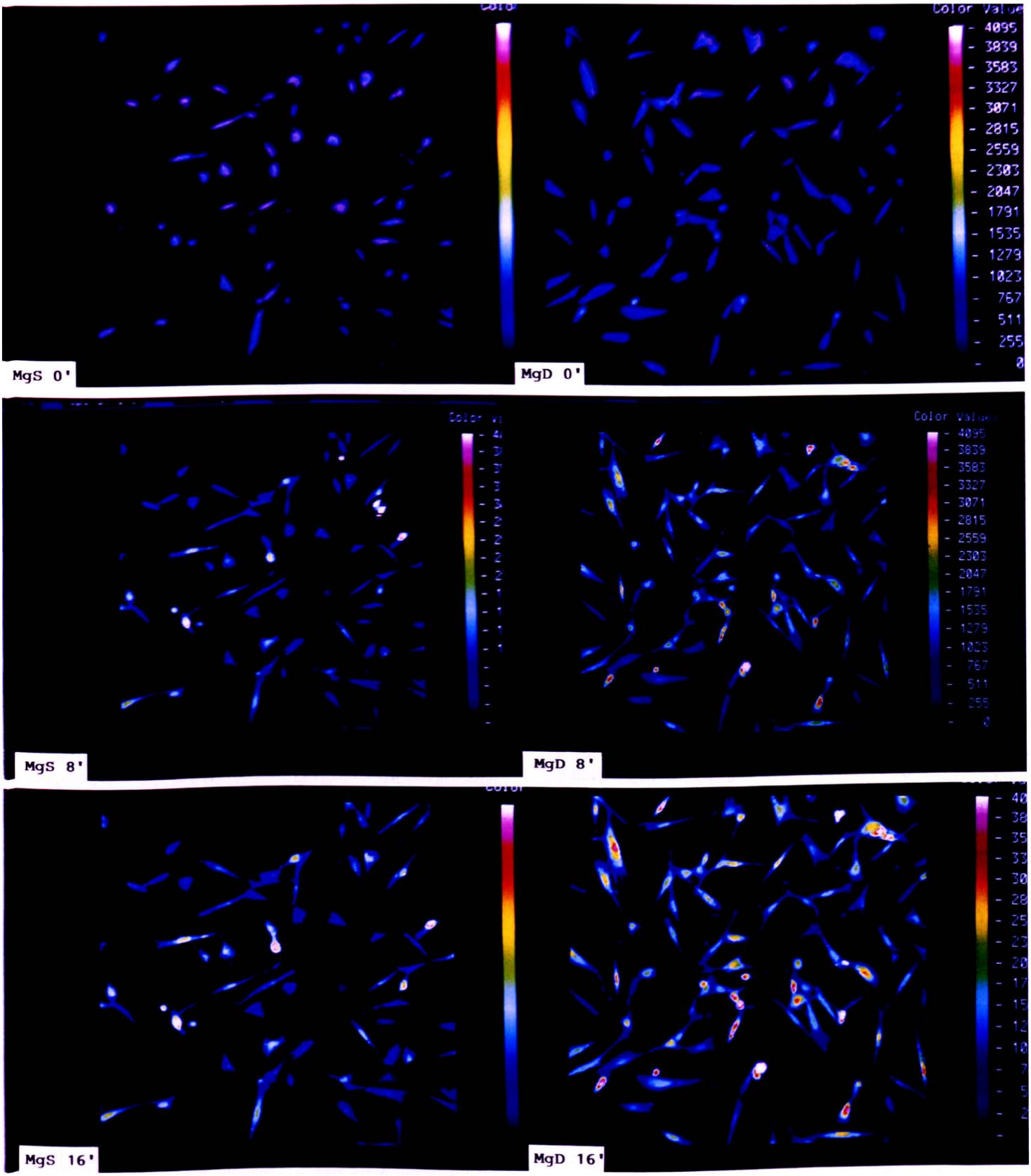
Fig. 2. Effect of culture Mg on the production of fluorescent 2,7-dichlorofluorescin with DCFA-labelled endothelial cells. Cells were grown in either 0.4 mM (right panel) or 0.8 mM Mg (left panel). Pseudocolor fluorescence intensity is shown for paired exposures times to free radicals: 0 min (top), 8 min (middle) and 16 min (lower).


MgS 0'
MgD 0'
MgS 8'
MgD 8'
MgS 16'
MgD 16'

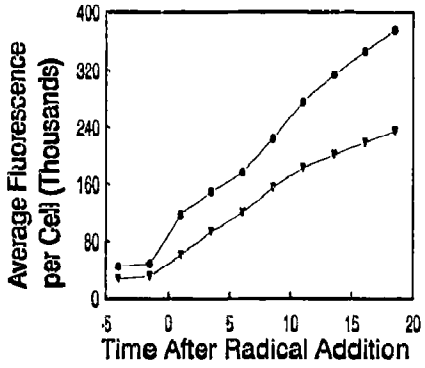


Fig. 3. Time-course of free radical-induced lipid peroxidation in attached endothelial cells grown in 0.4 mM (●) and 0.8 mM Mg. Endothelial cells grown on 22 mm coverslips were labeled with 2,7-dichlorofluorescin diacetate then exposed to the free radical system. Cellular fluorescence was obtained and quantified using the ACAS 570 Interactive Laser Cytometer.

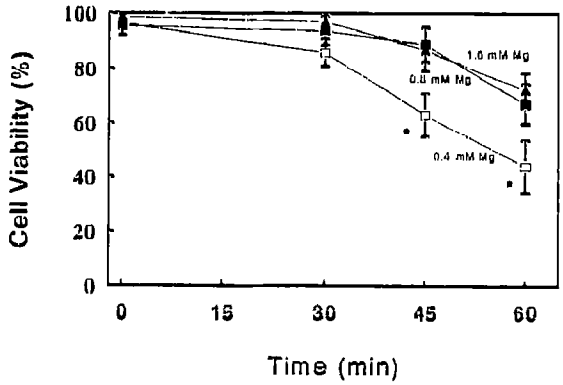


Fig. 4. Effect of culture Mg on free radical-induced endothelial cell killing. Cells were grown in either 0.4 mM (□), 0.8 mM Mg (■), or 1.6 mM Mg (▲) and exposed to the free radical system. Cell viability was determined by Trypan blue (0.1%) exclusion. $^{*}P < 0.05$ compared to 0.8 mM grown cells.

which endothelial cells cultured in low and normal Mg were exposed to the hydroxyl radical generating system. The photographs are digitized images with fluorescence intensity shown in pseudocolor. The color scale is from black and dark blue as low intensity, to purple and white as highest fluorescence intensity. In this figure, endothelial cells cultured in low Mg displayed dramatically more intracellular oxidation in response to exogenous free radicals than cells grown in sufficient Mg. The average fluorescence per cell in this experiment is shown in Fig. 3. In most experiments, upon exposure to the exogenous free radicals the cellular fluorescence initially increased and was followed by a rapid loss of fluorescence in cells cultured under both Mg-sufficient and Mg-deficient conditions. However, even in these cases, as represented in Fig. 3, the increase in fluorescence usually occurred more rapidly in Mg-deficient cells than in the paired Mg-sufficient cells. The addition of Trypan blue immediately after the loss of cellular fluorescence, while the sample was still on the microscope stage, demonstrated that the loss of fluorescence was not associated with a loss of cellular viability. Further time-course studies of the effect of Mg deficiency on free radical-induced loss of cellular viability were assessed; the results in Fig. 4 demonstrated that Mg-deficient cells were 2-fold more susceptible to free radical cytotoxicity. Culturing cells with a higher level of Mg (1.6 mM) failed to provide additional protection against free radical-induced cytotoxicity.

Our laboratory has shown that Mg deficiency increases the circulating inflammatory cytokines [8], and presumably neutrophil-mediated free radical production. Mg deficiency also has been reported to increase serum iron [6]. Our use of DHF and FeADP mimics the endogenous oxidative stress endothelial cells would encounter from these blood components. The enhanced susceptibility towards oxidative injury of attached Mg-deficient cells suggests that the differences seen in harvested cells are related to culturing in low Mg and not to potential injury during cell harvesting. The causes of this enhanced oxidative injury remain unclear. Culturing in low Mg results in a change in the endothelial cell lipid composition, which might predispose the cells to oxidative injury. We have obtained preliminary data indicating that culturing endothelial cells in low Mg medium, for 5–7 days, resulted in slightly lower glutathione levels. However, when cells were subjected to free radical stress, the glutathione decreased much more rapidly in the Mg-deficient cells compared to the Mg-sufficient cells. The rapid loss of glutathione in Mg-deficient cells is consistent with a higher level of peroxide formation and subsequent increased consumption of intracellular glutathione [10,17]. While the exact molecular mechanisms remain to be determined, the present study clearly indicates that endothelial cells grown in low Mg are more susceptible to oxyradical-mediated intracellular lipid peroxidation. The results also suggest that endothelial oxidative injury may contribute to increased vascular injury during Mg deficiency.

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REFERENCES

[1] Weglicki, W. B., Freedman, A.M., Bloom, S., Atrakchi, A.H., Cassidy, M.M., Dickens, B.F. and Mak, I.T. (1992) Am. J. Cardiovasc. Pathol. (in press).
[2] Atrakchi, A.H., Bloom, S., Dickens, B.F., Mak, I.T. and Weglicki, W.B. (1992) J. Cardiovas. Pathol. 1, 155–160.
[3] Freedman, A.M., Atrakchi, A.H., Cassidy, M.M. and Weglicki, W.B. (1990) Biochem. Biophys. Res. Commun. 170, 1102–1106.
[4] Freedman, A.M., Mak, I.T., Stafford, R.E., Dickens, B.F., Cassidy, M.M., Muesing, R. and Weglicki, W.B. (1992) Am. J. Physiol. 262, C1371–C1375.

[5] Freedman, A.M., Cassidy, M.M. and Weglicki, W.B. (1991) Magnesium Res. 4, 185–189.
[6] Itokawa, Y. (1987) Magnesium 6, 48–54.
[7] Altura, B.T., Brust, M., Bloom, S., Barbour, R.L., Stempak, J.G. and Altura, B.M. (1990) Proc. Natl. Acad. Sci. USA 87, 1840–1844.
[8] Weglicki, W.B., Phillips, T.M., Freedman, A.M., Cassidy, M.M. and Dickens, B.F. (1992) Mol. Cell. Biochem. 110, 169–173.
[9] Mak, I.T., Freedman, A.M., Dickens, B.F. and Weglicki, W.B. (1990) Biochem. Pharmacol. 40, 2169–2175.
[10] Mak, I.T., Boehme, P. and Weglicki, W.B. (1992) Circ. Res. 70, 1099–1103..
[11] Mak, I.T. and Weglicki, W.B. (1991) Circulation 84 (II) 256.
[12] Kramer, J.H., Mak, I.T. and Weglicki, W.B. (1984) Circ. Res. 55, 120–124.
[13] Mak, I.T., Kramer, J.H., Freedman, A.M., Tse, S.Y.H. and Weglicki, W.B. (1990) J. Mol. Cell. Cardiol. 22, 687–695.
[14] Banai, S., Haggroth, L., Epstein, S.E. and Casscells, W. (1990) Circ. Res. 67, 645–650.
[15] Beckman, J.S., Minor Jr., R.L., White, C.W., Repine, J.E., Rosen, G.M. and Freeman, B.A. (1988) J. Biol. Chem. 263, 6884–6892.
[16] Kuzuya, M., Naito, M., Funaki, C., Hayashi, T., Asai, K. and Kuzuya, F. (1989) Biochem. Biophys. Res. Commun. 163, 1466–1472.
[17] Mak, I.T., Dickens, B.F., Andle, K., Boehme, P. and Weglicki W.B. (1992) FASEB J. 6, 2357.