Intracellular Ca^{2+} dependence of nitric oxide mediated enhancement of interleukin-8 secretion in human endothelial cells

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Abstract Nitric oxide (NO•) can induce transient $[Ca^{2+}]$ changes in endothelial cells not different from receptor mediated signalling. Whether this Ca^{2+} signal may provide a link with IL-8 secretion induced by NO• donors was investigated in human endothelial cells. Sodium nitroprusside (SNP) and *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) dose dependently increased IL-8 production in this cell type. Additive IL-8 secretion was found with TNFα. Buffering intracellular Ca^{2+} with MAPT/AM suppressed NO• induced $[Ca^{2+}]_i$ changes and reduced subsequent IL-8 secretion. The additive effect of both NO• donors on TNFα induced IL-8 secretion was completely blocked in the presence of MAPT/AM. SKF 96365, which has been shown to block receptor mediated Ca^{2+} entry, and TMB-8, which blocks intracellular Ca^{2+} release, both inhibited IL-8 secretion, particularly when TNFα was used as a costimulator, indicating that $[Ca^{2+}]_i$ changes are important components of IL-8 induction by NO•.

Key words: Nitric oxide; Cytoplasmic Ca^{2+} signal; Interleukin-8; Endothelium

1. Introduction

Interleukin 8 (IL-8) is a neutrophil chemoattractant produced from a variety of cells including monocytes and endothelial cells [1]. Increased plasma and cell associated levels of this cytokine were found in patients suffering from sepsis, trauma and acute respiratory distress syndrome [2,3]. Moreover, the application of anti-IL-8 provided significant protection in several animal models of these diseases [4,5]. Together with IL-8 an increased production of nitric oxide has also been recognized in the same pathological states [6–8]. The potential of nitric oxide to induce IL-8 gene transcription has recently been demonstrated [9–12]. However, the mechanisms have not been addressed. Several reports led us to hypothesize that cytoplasmic $[Ca^{2+}]$ changes may provide a link for these observations. Ca^{2+} ionophores like A23187 and ionomycin induce or augment IL-8 secretion in different cell types [13,14]. Recently we have shown that nitric oxide is able to induce transient $[Ca^{2+}]_i$ changes in endothelial cells similar to receptor mediated changes proximal or at the IP_3 receptor [15]. We therefore investigated whether $[Ca^{2+}]_i$ changes induced by NO• donors contribute to IL-8 production in endothelial cells.

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Abbreviations: IL-8, interleukin 8; TNFα, tumor necrosis factor α; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine; SNP, sodium nitroprusside

2. Materials and methods

2.1. Cell culture

Human endothelial cell line ECV304 (ECACC, Cerdic, Sophia Antipolis, France) was cultured in RPMI medium (Sigma, Deisenhofen, Germany) at 37°C (21% O_2/5% CO_2), supplemented with fetal calf serum (10%, Sigma), glutamine (2 mM, Gibco, Eggenstein, Germany), penicillin (100 U/ml, Sigma), streptomycin (100 μg/ml), and grown in culture flasks (Falcon, Heidelberg, Germany).

2.2. $[Ca^{2+}]_i$ and IL-8 measurements

$[Ca^{2+}]_i$ measurements were performed in buffer which consisted of 127 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 10 mM D-glucose, pH 7.3, plus 1.8 mM $CaCl_2$. Subconfluent cells growing on glass coverslips were rinsed 4 times with buffer. $[Ca^{2+}]_i$ was measured in single cells loaded for 30 min with 1 μM of the calcium sensitive probe FURA-2/AM (Molecular Probes, Eugene, OR, USA) dissolved in dimethylformamide. For cell imaging a digital analysis system (T.I.L.L. photonics, Planegg, Germany) connected to an inverted microscope (DMIRB, Leica) equipped with a Zeiss Fluar 40×/1.30 oil objective was used. Excitation wavelengths (340 nm, 380 nm) were produced by a scanning monochromator and images of fluorescence passing a 395 nm dichroic mirror and a 520 ± 10 nm band pass filter were recorded by a cooled slow scan CCD camera at a rate of 2 Hz. Given are data for fluorescence ratio values (excitation 340 nm/380 nm) [15,16]. For the measurement of IL-8 cells were cultured for 24 h in 96 multiwell plates (Falcon). IL-8 in the supernatant was measured using a commercially available ELISA (Milenia, DPC, Bad Nauheim, Germany).

2.3. Cytotoxicity assay

S-Nitroso-*N*-acetyl-DL-penicillamine (SNAP; Calbiochem, Germany), sodium nitroprusside (SNP; Merck, Darmstadt, Germany) up to the highest concentrations used in this study (1 mM), SKF 96365 (Calbiochem-Novabiochem, Bad Soden/Ts., Germany), TMB-8 (Calbiochem-Novabiochem), MAPT/AM (Molecular Probes) did not result in increased trypan blue uptake compared to control cells to indicate viability [17].

2.4. Statistical analysis

To compare the measured results in different groups descriptive statistical parameters (mean ± S.E.M.) were calculated and differences were tested for significance using an ANOVA. The Student-Newman-Keuls test was used to compare individual results with the control. P values < 0.05 were considered to be significant.

3. Results

3.1. Nitric oxide donors induce transient $[Ca^{2+}]_i$ changes in endothelial cells

In a previous study, we showed that NO• donors transiently increase $[Ca^{2+}]_i$ in endothelial cells indistinguishable from receptor mediated changes [15]. This transient increase upon addition of both SNP (100 μM; increase in fluorescence ratio units from 34.8 ± 4.2 to 163.6 ± 16.7; $P < 0.001$) and SNAP (100 μM; peak fluorescence ratio units: 174.0 ± 10.7 compared to baseline levels of 35.7 ± 4.3; $P < 0.001$) (Fig. 1A,B) is prevented in the presence of MAPT/AM (15 μM;

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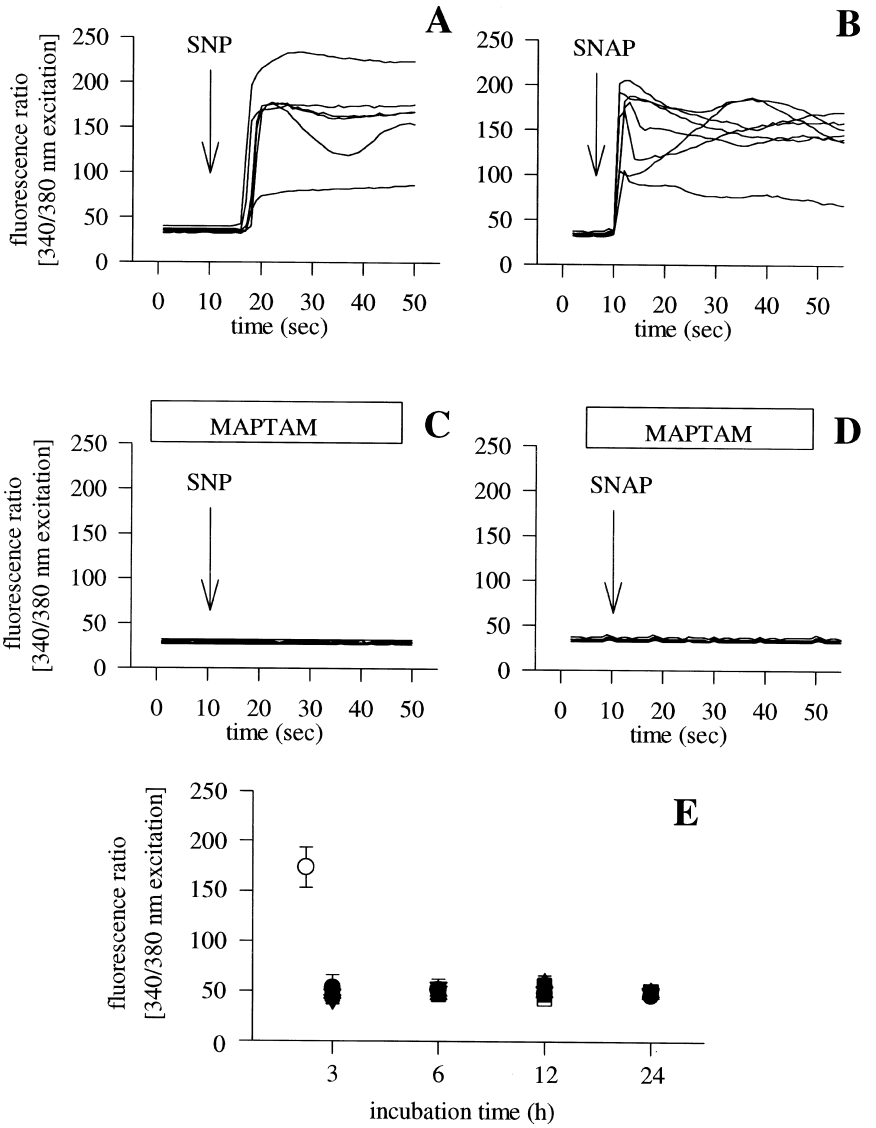

Fig. 1. $[Ca^{2+}]_i$ changes in single endothelial cells induced by nitric oxide donors and TNFα. A,B: Cells were treated with SNP (100 μM) or SNAP (100 μM) as indicated by the arrow and FURA-2 signals were recorded in at least six cells simultaneously. C,D: Cells were pretreated with MAPT/AM (15 μM) for 15 min and stimulated with SNP (100 μM) or SNAP (100 μM) as in A,B. Represented are typical measurements of four independent experiments in each setting. E: Influence of long term exposure of endothelial cells with SNP (100 μM; ○), TNF (10 ng/ml; △), SNP+TNF (▽), SNP+MAPT/AM (15 μM; ■), TNF+MAPT/AM (▲), TNF+SNP+MAPTAM (▼). SNP (100 μM) short time treatment (○) is shown in comparison. Symbols represent means ± S.E.M. of experiments performed in triplicate.

$P < 0.001$) (Fig. 1C,D). $[Ca^{2+}]_i$ levels of unpretreated cells stimulated with SNP (100 μM) or SNAP (100 μM) return to baseline within 5–10 min and incubations with SNP (100 μM), SNAP (100 μM), TNFα (10 ng/ml), MAPT/AM (15 μM) and combinations thereof did not induce any increased $[Ca^{2+}]_i$ levels up to 24 h in the continuous presence of these substances (Fig. 1E).

3.2. *NO• donating drugs induce secretion of IL-8*

IL-8 secretion from ECV 304 cells was measured in the supernatant after 24 h of incubation with NO• donating agents in the presence or absence of oxyhemoglobin to scavenge nitric oxide. SNP (10 μM–1 mM) and SNAP (10 μM–1 mM) induced a concentration dependent secretion of IL-8 (compared to untreated controls $P < 0.001$ for 100 μM and 1 mM for both substances; Fig. 2). Since we were able to show previously that concentrations of SNP above 5 mM and SNAP above 1 mM cause toxic damage to our cells [17] in all further experiments we used 100 μM of both NO• donors. In the presence of oxyhemoglobin (50 μM) IL-8 secretion was not different from untreated control cells for both SNP (100 μM) and SNAP (100 μM) indicating that NO• was the primary mediator (Fig. 2).

3.3. *Secretion induced by TNFα is enhanced by NO• donating agents*

TNFα induced IL-8 secretion in endothelial cells after 24 h (3366 ± 568 pg/ml) was found to be stronger than IL-8 secretion induced by either 100 μM SNP (2892 ± 397 pg/ml) or 100 μM SNAP (2156 ± 255 pg/ml). However, simultaneous administration of either SNP plus TNFα or SNAP plus TNFα increased IL-8 production by endothelial cells (4702 ± 392 pg/ml and 5439 ± 210 pg/ml, respectively) (Fig. 3).

3.4. Role of $[Ca^{2+}]_i$ in IL-8 secretion induced by NO• donors

Whether $[Ca^{2+}]_i$ changes induced by SNP or SNAP contribute to IL-8 secretion was tested in the presence of the Ca^{2+} buffer MAPT/AM. In this series the induction of IL-8 secretion after 24 h by 100 μM SNP (3122 ± 465 pg/ml) was only marginally reduced in the presence of MAPT/AM (2882 ± 526 pg/ml) whereas secretion induced by 100 μM SNAP (2156 ± 255 pg/ml) was reduced in the presence of MAPT/AM (1327 ± 194 pg/ml; $P < 0.05$). However, IL-8 production induced by combined treatment with 10 ng/ml TNFα plus 100 μM SNP (4616 ± 470 pg/ml) or 10 ng/ml TNFα plus 100 μM SNAP (4322 ± 503 pg/ml) was significantly reduced to baseline production in the presence of MAPT/AM (1635 ± 136 pg/ml and 1466 ± 205 pg/ml, $P < 0.01$ each, respectively) (Fig. 4A). Drugs inhibiting Ca^{2+} release from intracellular stores (TMB-8) [18] or receptor mediated capacitative Ca^{2+} entry (SKF 96365) [19] were also tested. In these series the presence of TMB-8 decreased SNP (100 μM) induced IL-8 secretion from 3122 ± 465 pg/ml to 758 ± 44 pg/ml ($P < 0.05$) and secretion induced by 100 μM SNP plus 10 ng/ml TNFα from 4616 ± 470 pg/ml to 956 ± 153 pg/ml ($P < 0.01$). The presence of SKF also reduced SNP (100 μM) induced IL-8 secretion to 1704 ± 357 pg/ml ($P < 0.05$) and SNP (100 μM) plus TNFα (10 ng/ml) induced IL-8 secretion to 1970 ± 525 pg/ml ($P < 0.05$) (Fig. 4B).

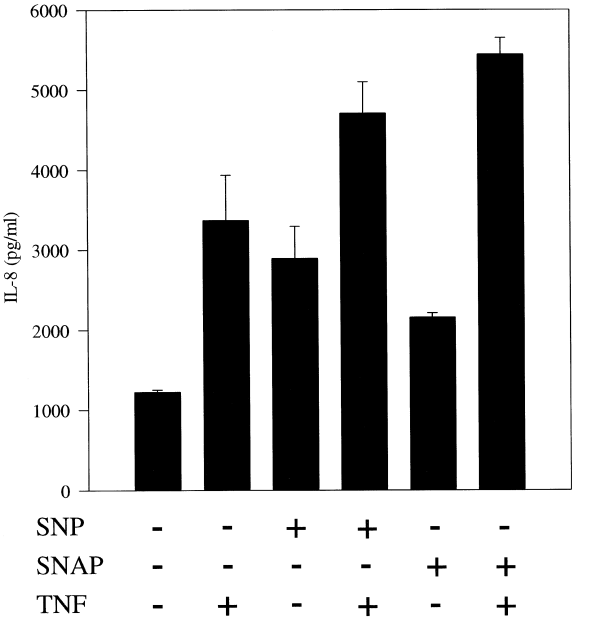

Fig. 3. NO• donors increase IL-8 production induced by TNF. SNP (100 μM), SNAP (100 μM) and TNF (10 ng/ml) were added to endothelial cell cultures as indicated. The graph represents mean ± S.E.M. values of supernatant IL-8 of three independent experiments performed in duplicate.

4. Discussion

Several groups demonstrated the potential of nitric oxide to regulate proinflammatory cytokine expression [20–23]. In particular, IL-8 expression may well be supported by NO• overproduction in vivo [6]. This cytokine has also been reported to be inducible by Ca^{2+} ionophores like A23187 and ionomycin [13,14]. Intracellular $[Ca^{2+}]$ changes have long been known to control stimulus-secretion coupling. However, participation in proinflammatory gene transcription has also been demonstrated [24]. For example, LPS induced IL-1 secretion has been shown to be Ca^{2+} dependently regulated [25]. Moreover, Ca^{2+} dependent gene transcription in neuronal cells has been reported to be enhanced by NO• [26]. $[Ca^{2+}]_i$ changes may therefore serve as a critical link in NO• mediated IL-8 secretion. In a previous study we demonstrated that nitric oxide induces transient $[Ca^{2+}]_i$ changes similar to receptor mediated changes in endothelial cells [15]. In the present study we show that transient $[Ca^{2+}]_i$ changes induced by NO• donors may provide at least part of the signal for IL-8 secretion. Induction of IL-8 by two different NO• donating agents was blocked by pre-incubation with oxyhemoglobin, the most important proteinaceous trap of NO• in vivo [27]. Likewise, $[Ca^{2+}]_i$ changes induced by these NO• donors were also blocked by oxyhemoglobin as has been demonstrated previously [15]. Experiments using MAPT/AM to buffer intracellular Ca^{2+} ions inhibited $[Ca^{2+}]_i$ changes but IL-8 secretion was only marginally reduced in the case of SNP, whereas SNAP induced IL-8 secretion was completely suppressed. However, IL-8 production induced by both NO• donors in TNFα treated cells was most effectively blocked in the presence of MAPT/AM. Inhibition of Ca^{2+} release from intracellular stores by TMB-8 and inhibition of Ca^{2+} entry by SKF 96365 also reduced IL-8 secretion in SNP and TNFα plus SNP treated cells. In conclusion these results indicate that NO• induced $[Ca^{2+}]_i$ changes may serve as a critical signal for IL-8 secretion, particularly in the presence of TNFα. Whether drugs affecting Ca^{2+} regulation are of benefit in complex situations where proinflammatory cytokines and nitric oxide are overproduced awaits further investigation [28].

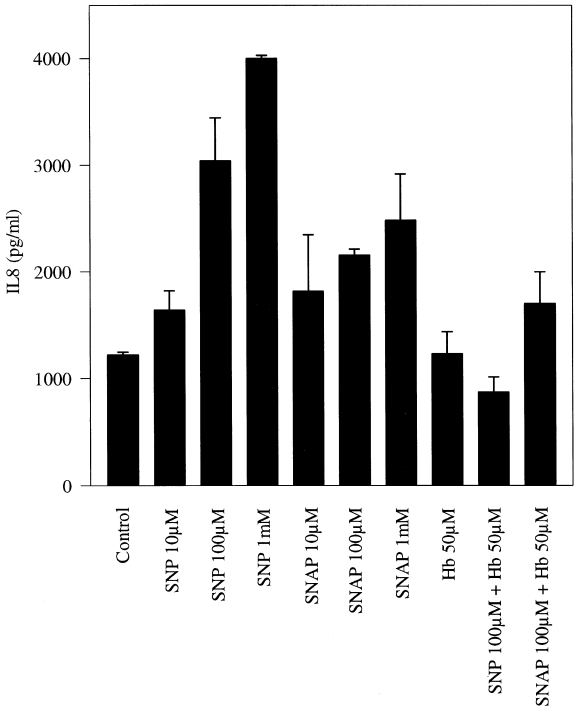

Fig. 2. IL-8 production from endothelial cells stimulated with NO• donors. Endothelial cells were stimulated with the indicated substances for 24 h. Shown are mean ± S.E.M. values of supernatant IL-8 of three independent experiments performed in duplicate.

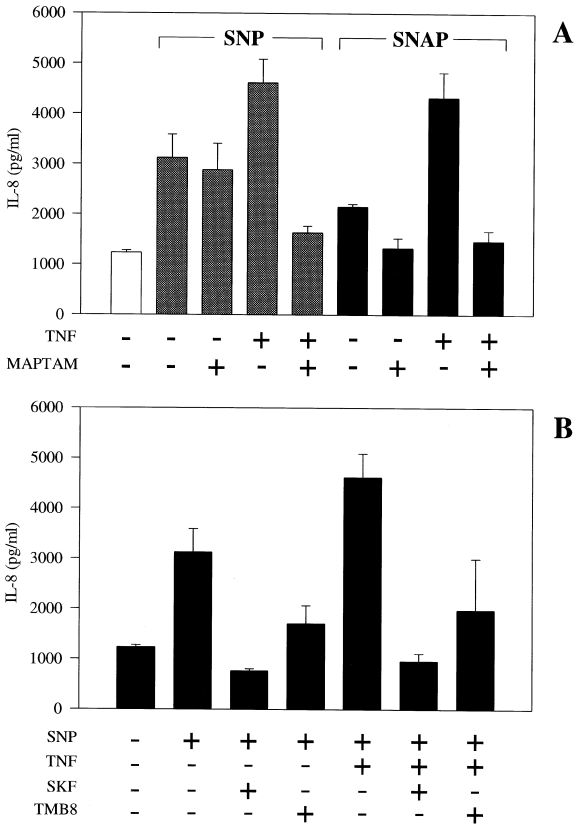

Fig. 4. Influence of drugs affecting [Ca^{2+}] regulation on NO• donor induced IL-8 secretion from endothelial cells. A: Cells were treated for 24 h with SNP (100 μM; crosshatched bars) or SNAP (black bars) in the presence of TNF (10 ng/ml) and/or MAPT/AM as indicated. B: Cells were treated with SNP (100 μM), TNF (10 ng/nl), SKF 96365 (50 μM), and TMB-8 (100 μM) for 24 h as indicated. The graph represents mean ± S.E.M. values of supernatant IL-8 of two independent experiments performed in duplicate.

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References

[1] Baggiolini, M., Dewald, B. and Moser, B. (1994) Adv. Immunol. 55, 97–179.
[2] Marie, C., Fitting, C., Cheval, C., Losser, M.R., Carlet, J., Payen, D., Foster, K. and Cavaillon, J.M. (1997) Infect. Immun. 65, 865–871.
[3] Grau, G.E., Mili, N., Lou, J.N., Morel, D.R., Ricou, B., Lucas, R. and Suter, P.M. (1996) Lab. Invest. 74, 761–770.
[4] Sekido, N., Mukaida, N., Harada, A., Nakanishi, I., Watanabe, Y. and Matsushima, K. (1993) Nature 365, 654–657.
[5] Yokoi, K., Mukaida, N., Harada, A., Watanabe, Y. and Matsushima, K. (1997) Lab. Invest. 76, 375–384.
[6] Kobayashi, A., Hashimoto, S., Kooguchi, K., Tanaka, Y., Oonodera, H., Urata, Y. and Ashihara, T. (1997) Acta Histochem. Cytochem. 30, 57–61.
[7] Fink, M.P. and Payen, D. (1996) Intens. Care Med. 22, 158–165.
[8] Ochoa, J.B., Udekwu, A.O., Billiar, T.R., Curran, R.D., Cerra, F.B., Simmons, R.L. and Peitzman, A.B. (1991) Ann. Surg. 214, 621–626.
[9] Cuthbertson, B.H., Galley, H.F. and Webster, N.R. (1997) Br. J. Anaesth. 78, 714–717.
[10] Brown, Z., Robson, R.L. and Westwick, J. (1993) Adv. Exp. Med. Biol. 351, 65–75.
[11] Villarete, L.H. and Remick, D.G. (1995) Biochem. Biophys. Res. Commun. 211, 671–676.
[12] Villarete, L.H. and Remick, D.G. (1997) Shock 7, 29–35.
[13] Braun, R.K., Franchini, M., Erard, F., Rihs, S., De Vries, I.J., Blaser, K., Hansel, T.T. and Walker, C. (1993) Eur. J. Immunol. 23, 956–960.
[14] Wilson, L., Butcher, C.J. and Kellie, S. (1993) FEBS Lett. 325, 295–298.
[15] Volk, T., Mäding, K., Hensel, M. and Kox, W.J. (1997) J. Cell. Physiol. (in press).
[16] Graier, W.F., Simecek, S., Kukovetz, W.R. and Kostner, G.M. (1996) Diabetes 45, 1386–1395.
[17] Volk, T., Ioannidis, I., Hensel, M., deGroot, H. and Kox, W.J. (1995) Biochem. Biophys. Res. Commun. 213, 196–203.
[18] Yamada, Y., Teraoka, H., Nakazato, Y. and Ohga, A. (1988) Neurosci. Lett. 90, 338–342.
[19] Graier, W.F., Groschner, K., Schmidt, K. and Kukovetz, W.R. (1992) Biochem. Biophys. Res. Commun. 186, 1539–1545.
[20] Eigler, A., Sinha, B. and Endres, S. (1993) Biochem. Biophys. Res. Commun. 196, 494–501.
[21] Van Dervort, A.L., Yan, L., Madara, P.J., Cobb, J.P., Wesley, R.A., Corriveau, C.C., Tropea, M.M. and Danner, R.L. (1994) J. Immunol. 152, 4102–4109.
[22] Lander, H.M., Sehajpal, P., Levine, D.M. and Novogrodsky, A. (1993) J. Immunol. 150, 1509–1516.
[23] Marcinkiewicz, J., Grabowska, A. and Chain, B. (1995) Eur. J. Immunol. 25, 947–951.
[24] Chin, K.V., Cade, C., Brostrom, C.O., Galuska, E.M. and Brostrom, M.A. (1987) J. Biol. Chem. 262, 16509–16514.
[25] Ohmori, Y. and Hamilton, T.A. (1992) J. Immunol. 148, 538–545.
[26] Peunova, N. and Enikolopov, G. (1993) Nature 364, 450–453.
[27] Kosaka, H., Uozumi, M. and Tyuma, I. (1989) Free Radical Biol. Med. 7, 653–658.
[28] Hughes, C.B., el Din, A.B., Kotb, M., Gaber, L.W. and Gaber, A.O. (1996) Pancreas 13, 22–28.