In vitro cytotoxic effects of tumor necrosis factor-α in human breast cancer cells may be associated with increased glucose consumption

Ofer Kaplan[a,b,*], Jesús Ruíz-Cabello[c], Jack S. Cohen[1,b]

[a] *Department of Surgery A, Tel-Aviv Medical Center, 6 Weizmann St., Tel-Aviv 64239, Israel*
[b] *Cancer Pharmacology Section, Georgetown University Medical School, Washington, DC, USA*
[c] *Department of Physical Chemistry, NMR Unit, Faculty of Pharmacy, UCM, Madrid, Spain*

Received 22 January 1997

Abstract Tumor necrosis factor-α inhibited growth of cultured MCF-7 human breast cancer cells in a dose dependent manner. Tumor necrosis factor-α also markedly increased glucose consumption, and its cytotoxicity was modified by glucose concentrations in the growth medium; higher glucose levels were associated with increased cell survival. However, when the cells were perfused in physiological conditions, very high levels of tumor necrosis factor-α (200 ng/ml) in the perfusion solution had no inhibitory effects. Moreover, tumor necrosis factor-α had no effects on ^{31}P nuclear magnetic resonance spectra of the perfused cells. In the traditional growth inhibition assays, cells are incubated for several days with a drug, a situation where their metabolism is altered due to the depletion of nutrients, the accumulation of toxic waste materials and pH changes. Perfusion experiments are more relevant to in vivo conditions, and may be used for studying metabolic processes and the mechanisms of action of therapeutic agents.

Key words: Tumor necrosis factor-α; Cancer cell; Nuclear magnetic resonance; Glucose consumption

1. Introduction

Tumor necrosis factor-α (TNF-α) is a polypeptide which has an extensive array of biological activities, and its effects are initiated by its binding to specific cell surface receptors [1,2]. TNF-α inhibits the growth of some, but not all, tumor cell lines, while sparing normal cells [3,4]. However, this selective inhibition is due to different signals generated by the cytokine, rather than the number of TNF-α receptors [3,5]. The cell-specific post-receptor mechanism of action of TNF-α is still ill-defined. It involves diverse and complex metabolic alterations, and metabolic inhibitors, such as cycloheximide and actinomycin D, enhance the TNF-α induced cytotoxicity [6,7].

NMR spectroscopy is a very valuable method for studying the metabolism of intact cells and tissues in a continuous non-invasive manner [8,9]. We used ^{31}P NMR to assess the effects of TNF-α on perfused MCF-7 human breast cancer cells in physiological conditions. Cytotoxicity and glucose consumption were also evaluated in cultured cells treated with TNF-α. Our data point to the pitfalls in the interpretations of results obtained in in vitro experiments.

*Corresponding author (at a). Fax: (972) (3) 6974621.

[1] *Present address:* Sheba Medical Center, Israel.

Abbreviations: TNF-α, tumor necrosis factor-α; IMEM, improved minimal Eagle's medium; NMR, nuclear magnetic resonance

2. Materials and methods

2.1. Cells and materials

MCF-7 human breast cancer cells from the National Institutes of Health [10] were grown in IMEM (11 mM glucose), supplemented with penicillin-streptomycin-nystatin, L-glutamine and 5% fetal calf serum, under 5% CO_2 environment. IMEM and supplements were purchased from Gibco (Grand Island, NY, USA). Chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA) unless otherwise indicated, and were of the highest available purity. Recombinant TNF-α was from Genzyme Co. (Cambridge, MA, USA).

2.2. Cell survival and glucose consumption assays

The effects of TNF-α on MCF-7 cells were studied in cell survival assays. 25×104 cells were plated in 25-cm^2 flasks in 10 ml of IMEM (11 mM glucose). Twenty-four hours later, the medium was replaced with new growth medium containing 4, 11 or 25 mM glucose with various concentrations of TNF-α. Cells were harvested after 2 or 5 days, and the surviving cells were counted (Coulter counter, Coulter Electronics, Hialeah, FL, USA). For glucose consumption assays, the cells were similarly plated, and when they reached $40\pm5\%$ confluency, the medium was changed to IMEM (11 mM glucose) with various TNF-α concentrations. After 2 days, samples of medium were taken for glucose measurements, and a cell count (in flasks plated only for this purpose) was performed. Twenty-four hours later, medium samples were withdrawn again, and all cells were harvested and counted.

2.3. Perfusion of cells and NMR spectroscopy

The essentials of cellular perfusion are that metabolic events are unhampered; thus, substrates and nutrients can be continuously furnished, and waste products removed, while stable pH levels and a temperature of 37°C are maintained. In the present studies we used the method of cellular embedding in sodium alginate micro-capsules [11], prepared in sterile conditions in a biological hood. MCF-7 cells were grown to 80–90% confluency (six experiments), harvested with 0.25% trypsin-0.05% EDTA, centrifuged at 4°C at $750\times g$ for 7 min, and washed twice with medium. $1.5–2\times10^8$ cells were used in each experiment, and the cellular pellet (1.0–1.2 ml) was mixed with an equal volume of 2.5% (w/w in PBS) sodium alginate. The mixture was manually extruded, under minimal pressure, through a 25-gauge needle, on the surface of a 0.1 M $CaCl_2$ solution. The small drops (approx. 1 mm diameter) gelled and were immediately washed three times in the growth medium. The capsules were isolated by decantation, transferred to a 10 mm screw cap NMR tube, and perfusion was promptly initiated. The perfusion solution was IMEM containing the 'physiological' concentration of glucose, 4 mM. The perfusion was performed through an insert with inlet and outlet tubes, and the volume of the perfusion chamber was 2 ml. A constant flow of 0.9 ml/min in a single pass mode was maintained by a peristaltic pump (Pharmacia P-3, Pharmacia LKB Biotechnology Inc.), and the temperature was maintained at 37°C. The effluent was collected for glucose and lactate assays. In each experiment control perfusion with ^{31}P NMR recording was carried out for 3 h, to ensure metabolic stability of the cells, before adding TNF-α to the perfusion solution. ^{31}P Spectra were recorded on a Varian XL-400 spectrometer at 162 MHz, using 3 s relaxation delays, 45° radio-frequency pulses, and 200 transients in each spectrum. Peak integrals were used for monitoring the effects of TNF-α on the spectra. Cellular viability was evaluated by the trypan blue dye at the end of each perfusion experiment.

PII S0014-5793(97)00265-2

2.4. Glucose and lactate determination

Glucose and lactate concentrations were determined in perchloric acid treated samples, immediately following collection. Glucose concentrations were measured by the hexokinase enzymatic assay, using coupled enzyme reaction catalyzed by hexokinase and glucose-6-phosphate dehydrogenase, and measuring the product, NADH, at 340 nm. Lactate levels were determined utilizing the formation of NADH (and pyruvate) from lactate in the presence of hydrazine (to trap formed pyruvate) and excess NAD. The reaction is catalyzed by lactate dehydrogenase, and the lactate concentrations were calculated from the absorbance at 340 nm. Absorbance measurements were performed with a Shimadzu UV-160 spectrophotometer (Shimadzu Co.).

Quantitative results are expressed as means ± S.D. Statistical analyses were performed with the paired, double-tailed, Student's *t*-test ($P < 0.05$).

3. Results and discussion

The effects of TNF-α on the growth of cultured MCF-7 cells are shown in Fig. 1. A dose dependent inhibition by TNF-α was demonstrated, and these effects were rather prompt as considerable growth inhibition was observed even after an incubation time of 2 days. The effects of TNF-α were modified however by glucose concentrations in the medium. Fifty percent growth inhibition was obtained after 2 days with TNF-α concentrations of $\gg 50$, 33 and 22 ng/ml for 25, 11 and 4 mM glucose, respectively, and 47, 17.5 and 8.5 ng/ml for 25, 11 and 4 mM glucose, respectively, after 5 days. The differences in the results of the growth inhibition assays between the various glucose concentrations were statistically significant in the two aforementioned time periods ($P < 0.05$). Thus, higher glucose levels provided protection against TNF-α-induced cytotoxicity.

The effects of TNF-α on glucose consumption by MCF-7 cells are shown in Fig. 2. Preliminary experiments showed that in the first 2 days after plating, cell numbers were too small to

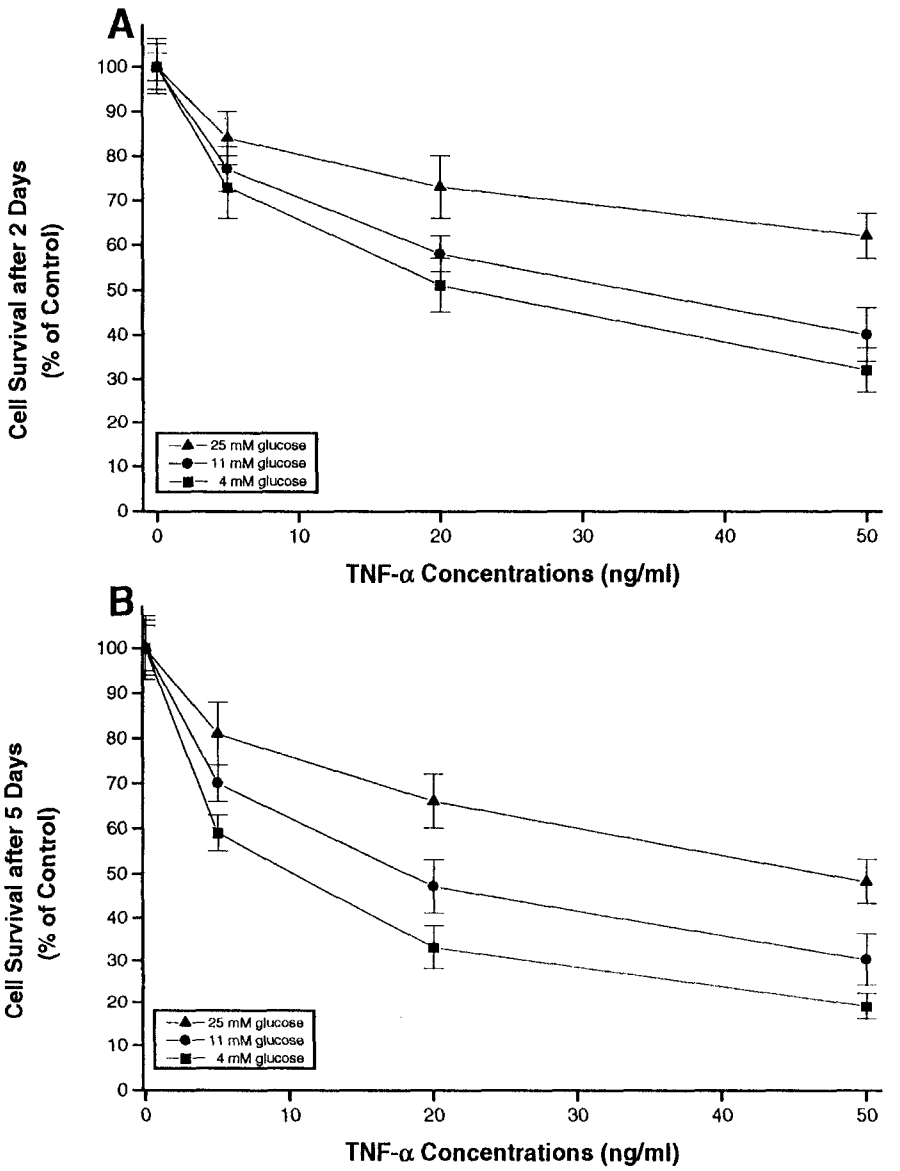

Fig. 1. Effects of TNF-α on the growth of MCF-7 cells cultured at different glucose concentrations. 25×10^4 cells were plated in 25-cm^2 flasks in 10 ml of IMEM (11 mM glucose). Twenty-four hours later, the medium was replaced with new growth medium with various concentrations of TNF-α. Surviving cells were counted after 2 (A) or 5 (B) days. The results were standardized to 100% of the control cells (without TNF-α). Mean ± S.D. of six measurements are presented.

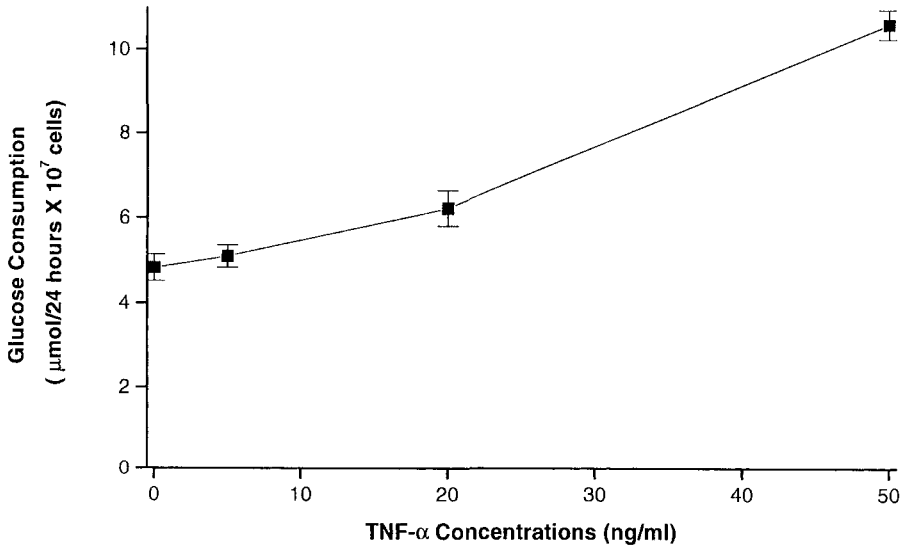

Fig. 2. Effects of TNF-α on glucose consumption by MCF-7 cells. 25×10^4 cells were plated in 25-cm^2 flasks in 10 ml of IMEM (11 mM glucose), and when they reached 40 ± 5% confluency, the medium was changed to IMEM (11 mM glucose) with various TNF-α concentrations. After 2 days, samples of medium were taken for glucose measurements, and a cell count (in flasks plated only for this purpose) was performed. Twenty-four hours later, medium samples were withdrawn again, and all cells were harvested and counted. Glucose utilization was calculated from the decrease in glucose concentrations over a period of 24 h relative to the average number of cells in that period. Mean ± S.D. of six measurements are presented.

detect changes in glucose concentrations accurately; therefore, glucose consumption was measured after 2 days. Glucose consumption was calculated from the changes in glucose concentrations over a period of 24 h, and is expressed relative to the average number of cells in that period. TNF-α markedly increased glucose consumption by MCF-7 cells, and at the concentration of 50 ng/ml the cells utilized 120% more glucose than untreated cells ($P < 0.03$). It should be noted that the MCF-7 cells were inhibited by TNF-α even in cultures where glucose was not completely depleted, and therefore, the toxicity of TNF-α cannot be solely attributed to lack of glucose and cell starvation.

^{31}P NMR spectra of perfused MCF-7 cells are shown in Fig. 3. Peaks were assigned according to previously reported data [12]. Addition of TNF-α (20, 50 and 200 ng/ml) to the perfusion solution caused no spectral changes. In this system, dead cells result in signal reduction, but even 48 h of perfusion with very high concentration of TNF-α (200 ng/ml, Fig. 3B), there was neither signal loss nor ATP depletion. It is noteworthy that in our previous NMR studies when cells were treated with metabolic inhibitors or cytotoxic agents ATP depleted preceding cell death [12,13]. Moreover, trypan blue exclusion dye examinations of the cells at the end of these prolonged perfusions demonstrated 90–95% cellular viability.

The perfusion system was also used for simultaneous NMR and glucose consumption and lactate production studies, thus correlating the energy status with glucose metabolism under physiological conditions. The results of these experiments are presented in Table 1. In the control experiments, the cells were perfused with IMEM (4 mM glucose), and glucose and lactate levels, which were measured in the effluent every 3 h, were stable for a perfusion period of 12 h. In the TNF-α experiments, cells were perfused as described before for 3 h, and samples were withdrawn for glucose and lactate determinations, followed by the addition of TNF-α (200 ng/ml) to the perfusion solution, and glucose and lactate were measured after 3 h of perfusion with TNF-α. TNF-α induced significant increase in glucose utilization and lactate production ($P < 0.05$, for both glucose and lactate). It is noteworthy though that the increase in glucose consumption exceeded the increase in lactate production. This may indicate that TNF-α induced also a change in the energy metabolism towards oxidative respiration, or that the products of glucose metabolism were used for the synthesis of protein and DNA. The marked increase in glucose consumption (elevation of more than 100% compared to control) caused very low glucose levels in the medium. However, there were no deleterious effects on the cells under these conditions as observed by the NMR spectra and the trypan blue examinations, probably due to the continuous supply of glucose and removal of waste products.

The cytotoxic mechanism of TNF-α is very complex and may induce both apoptotic and necrotic forms of cell lysis [2,14]. Oxygen is required for these processes [15], and it seems therefore that oxygen free radicals are involved in this mechanism. There is no unambiguous evidence that TNF-α directly hinders the synthesis of DNA, RNA or proteins. However,

Table 1
Effects of TNF-α on glucose and lactate levels in perfused MCF-7 cells

	Glucose (mM)	Lactate (mM)
Perfusion solution	3.96 ± 0.05	–
Effluent, control	2.65 ± 0.16	1.47 ± 0.12
Effluent with TNF	1.27 ± 0.13	2.34 ± 0.11

MCF-7 cells in alginate capsules were perfused with IMEM containing 4 mM glucose, at a constant rate of 0.9 ml/min, at 37°C. Following 3 h of initial perfusion, three samples of each perfusion solution and effluent were collected at 30 min intervals, and thereafter, TNF-α (200 ng/ml) was added to the perfusion solution. Three hours later, effluent samples were similarly collected. The means and S.D.s of three different experiments (total of nine measurements) are presented. In control experiments, glucose and lactate levels were stable for a perfusion period of 12 h. Lactate levels in the perfusion solution were negligible.

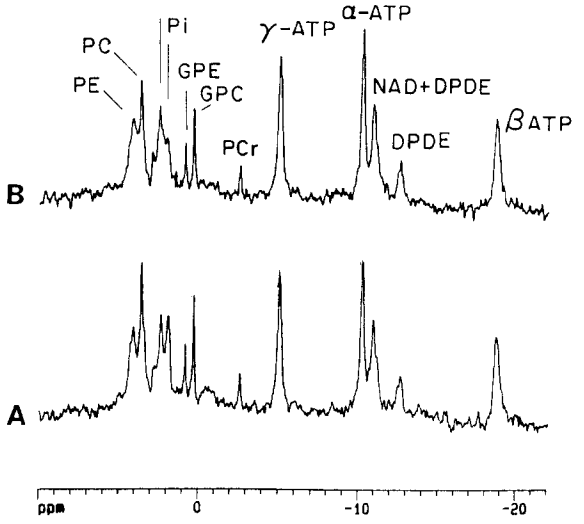

Fig. 3. ^{31}P NMR spectroscopy of perfused MCF-7 cells in alginate capsules. For perfusion conditions and acquisition parameters see Section 2. Line broadening of 15 Hz was used. Chemical shifts were determined by standardizing GPC to 0.49 ppm. PE, phosphoethanolamine; PC, phosphocholine; Pi, inorganic phosphate; GPE, glycerophosphoethanolamine; GPC, glycerophosphocholine; PCr, phosphocreatine; NAD, nicotineamid dinucleotide; DPDE, diphosphodiester. A, control; B, 48 h after adding TNF-α (200 ng/ml) to the perfusion solution (IMEM, 4 mM glucose).

inhibition of transcription or protein synthesis by metabolic inhibitors sensitizes cells to the cytotoxic action of TNF-α [4,6,7], suggesting that cells synthesize proteins that protect them from TNF-α effects.

The role of TNF-α in the treatment of cancer patients has been assessed in several clinical trials [16,17], but it appears that its use is limited because of severe systemic side effects, and wide variation in tumor sensitivity. Recently, TNF-α was used in isolated limb perfusion for the treatment of soft tissue sarcoma and malignant melanoma in combination with hyperthermia and conventional chemotherapy [18,19]. In solid tumors TNF-α may exert its effects through damage to the endothelium, and intravascular coagulation leading to ischemia, and not necessarily by direct toxicity against the tumor cells [20]. This mechanism may be particularly relevant to isolated limb perfusion therapy, and our finding that TNF-α was not toxic to perfused MCF-7 cells supports this hypothesis.

Persistent TNF-α production may causes cachexia, and it has been shown that TNF-α is identical to cachectin, which mediates the wasting of chronic infections [21]. The effects of TNF-α on energy and glucose metabolism are diverse, and were extensively studied in inflammation and septic shock models. In these situations TNF-α induces lipolysis, catabolism, and insulin resistance [21–23]. On the other hand, TNF-α increased glucose transport and utilization in several transformed, as well as normal, cell lines [24–27]. We found that TNF-α induced a dose dependent remarkable elevation of glucose consumption in MCF-7 human breast cancer cells. It is postulated that in cancer patients with very high levels of TNF-α, the much augmented glucose requirements are associated with the altered metabolism and cachexia.

Our studies demonstrated profound discrepancies between results obtained in cultured and perfused cells, and perhaps explain the inconsistent data regarding the effects of TNF-α in the laboratory and in the clinical setting. In the traditional growth inhibition assays cells are incubated for several days with a drug, a situation where their metabolism is altered due to the depletion of nutrients, the accumulation of toxic waste materials and pH changes. Perfusion methods are more relevant to in vivo conditions, and we suggest that this methodology should be used more often in experimental studies of physiology and metabolism, and in the development of therapeutic modalities.

References

[1] J. Vilcek, T.H. Lee, J Biol Chem 266 (1991) 7313–7316.
[2] R. Beyaert, W. Fiers, FEBS Lett 340 (1994) 9–16.
[3] B.J. Sugarman, B.B. Aggarwal, P.E. Hass, I.S. Figari, M.A. Palladino, H.M. Shepard, Science 230 (1985) 943–945.
[4] J.W. Larrick, S.C. Wright, FASEB J 4 (1990) 3215–3223.
[5] M. Tsujimoto, Y.K. Kip, J. Vilcek, Proc Natl Acad Sci USA 82 (1985) 7626–7630.
[6] V. Ruggiero, K. Latham, C. Baglioni, J Immunol 138 (1987) 2711–2717.
[7] T.R. Reid, F.M. Torti, G.M. Ringold, J Biol Chem 264 (1989) 4583–4589.
[8] Gadian DG. Nuclear Magnetic Resonance and its Application to Living Systems. New York: Oxford University Press, 1982:1170.
[9] O. Kaplan, J.S. Cohen, Breast Cancer Res Treat 31 (1994) 285–299.
[10] H.D. Soule, J. Vasquez, A. Long, S. Albert, M. Brennan, J Natl Cancer Inst 51 (1973) 1403–1409.
[11] K. Shankar-Narayan, E. Moress, J.C. Chatham, P.B. Barker, NMR Biomed 3 (1990) 23–26.
[12] O. Kaplan, G. Navon, R.C. Lyon, P.J. Faustino, E.J. Straka, J.S. Cohen, Cancer Res 50 (1990) 544–551.
[13] H. Ben-Horin, M. Tassini, A. Vivi, G. Navon, O. Kaplan, Cancer Res 55 (1995) 2814–2821.
[14] S.M. Laster, J.G. Wood, L.R. Gooding, J Immunol 141 (1988) 2629–2634.
[15] N. Matthews, M.L. Neale, S.K. Jackson, J.M. Stark, Immunology 62 (1987) 153–155.
[16] J.L. Abbruzzesa, B. Levin, J. Ajanai, Cancer Res 49 (1989) 4057–4061.
[17] P.B. Chapman, T.J. Lester, E.S. Casper, J Clin Oncol 5 (1987) 1942–1951.
[18] D. Lienard, F. Lejeune, J.J. Delmotte, N. Renard, P. Ewalenko, J Clin Oncol 10 (1992) 52–60.
[19] P.E. Sijens, A.M. Eggermont, P.V. van Dijk, M. Oudkerk, NMR Biomed 8 (1995) 215–224.
[20] N. Shine, M.A. Palladino, J.S. Patton, A. Deisseroth, G.S. Karczmar, G.B. Matson, M.W. Weiner, Cancer Res 49 (1989) 2123–2127.
[21] G. Camussi, E. Albano, C. Tetta, F. Bussolino, Eur J Biochem 202 (1991) 3–14.
[22] P. Sierra, P.R. Ling, N.W. Istfan, B.R. Bistrian, Metabolism 44 (1995) 1365–1370.
[23] Y. Sakurai, X.J. Zhang, R.R. Wolfe, Surgery 117 (1995) 305–313.
[24] S. Volland, E. Amtmann, G. Sauer, Int J Cancer 56 (1994) 698–705.
[25] P. Cornelius, M. Marlowe, P.H. Pekala, J Trauma 30 (12 Suppl) (1990) S15–20.
[26] N. Yu, D. Maciejewski-Lenoir, F.E. Bloom, P.J. Magistretti, Mol Pharmacol 48 (1995) 550–558.
[27] A. Zentella, K. Manogue, A. Cerami, Cytokine 4 (1993) 436–447.