Short Communication

Analysis of Sister-Chromatid Exchanges and Micronuclei in Cultured Human Lymphocytes Treated with Insulin

(insulin / lymphocyte / sister-chromatid exchange / micronucleus / mitotic index)

N. DJELIĆ

Department of Biology, Faculty of Veterinary Medicine, University of Belgrade, Belgrade, Yugoslavia

Abstract. Insulin is an anabolic hormone that may facilitate development of malignant diseases in various susceptible tissues due to stimulation of mitotic divisions. In this work, an evaluation of mitogenic and genotoxic effects of human recombinant insulin has been performed in cultures of human peripheral blood lymphocytes. Genotoxic effects were studied by the following test systems: (1) *in vitro* SCE test, and (2) cytokinesis-blocked micronucleus assay. The obtained results indicate that insulin stimulates mitotic division at an optimal concentration of 10^{-8} M. On the other hand, insulin has not exhibited genotoxic properties under experimental conditions in this investigation.

Insulin is a major anabolic hormone that induces metabolic changes via binding to specific membrane glycoprotein receptors detected in almost every cell type in mammals. Although the precise intracellular events that mediate the actions of insulin remain to be completely elucidated, the regulation of protein phosphorylation is thought to play an important role. Interestingly, insulin stimulates tyrosine phosphorylation of its receptors and induces subsequent cytobiochemical changes, which include an increase in activities of both phosphatases and kinases capable to target numerous intracellular proteins (Rosen, 1987; Dong et al., 2000).

An overwhelming evidence indicates that insulin stimulates progression through the cell cycle (Dahmer and Perlman, 1988), increases ^{3}H-thymidine incorporation (Menard and Dagenais, 1993) and causes mitogenic effects in numerous cell lines (Ish-Shalom et al., 1995; McIntyre et al., 1998). It is postulated that after insulin binds to its receptors, a signal cascade is triggered, modifying or inducing *de novo* synthesis of some proteins, which thereafter interact with target sequences on specific promoters, the so-called insulin response elements (O'Brien and Granner, 1991). In addition, upregulation of c-*fos* and c-*jun* protooncogenes (Peluso et al., 1995) may stimulate mitogenesis and facilitate tumor promotion in cells with genetic damage.

Despite the evidence of insulin action on gene expression, followed by cell growth and division, little is known about possible changes in nuclear genetic material in mammalian cells exposed to insulin. Jayanth et al. (1995) have shown that insulin inhibits repair of potentially lethal radiation damage, followed by an increased level of chromosome aberrations in pretreated human lung carcinoma cells. On the other hand, there is experimental evidence that mouse Swiss 3T6 cells, exposed to epidermal growth factor (EGF) and insulin immediately before irradiation, significantly restore single- and double-strand breaks (Bildin et al., 1990).

Having in mind numerous experimental data showing influence of insulin on the cell nucleus and its involvement in carcinogenesis, the objective of our study was to evaluate possible genotoxic effects in cultured human peripheral blood lymphocytes treated with a wide range of human recombinant insulin concentrations.

Material and Methods

Human peripheral blood lymphocyte cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan (1975). The cultures were established as previously described by Djelić et al. (1996). Heparinized whole blood samples (0.8 ml), obtained by venipuncture from three healthy men under 30 years of age, were added to vials with 9.2 ml of Parker 199 medium (Torlak, Belgrade, Yugoslavia) containing 30% of inactivated calf serum (Serva, Heidelberg, Germany) and 0.04 mg/ml of phytohemag-

Received June 13, 2000. Accepted August 28, 2000.

This investigation was supported by Serbian Ministry of Science and Technology, grant 12M18.

Corresponding author: Ninoslav J. Djelić, Department of Biology, Faculty of Veterinary Medicine, University of Belgrade, Bul. JNA 18, 11000 Belgrade, Serbia, Yugoslavia. Fax; Tel: + 381 11 685 936; e-mail:ndjelic@vet.bg.ac.yu.

Abbreviations: BrdUrd – 5-bromo-2'-deoxyuridine, CAS No. – Chemical Abstracts Service Number, FPG – fluorescence plus Giemsa, MI – mitotic index, MNNG – N-methyl-N'-nitro-N-nitrosoguanidine, SCE – sister-chromatid exchange.

glutinin (Murex Diagnostics Ltd., Dartford, England). Cultures were incubated for 72 h at (37 ± 0.5)°C.

Exactly 47 h and 30 min after the beginning of incubation, human recombinant insulin (CAS No. 11061-704, Humulin regular, Elli Lilly and Co., Indianapolis, IN) was added to the cultivation vials in such amounts as to obtain final experimental concentrations of 10^{-10} M, 10^{-9} M, 0.75×10^{-8} M, 2.5×10^{-8} M, 2.5×10^{-7} M, 2.5×10^{-6} M, 0.75×10^{-5} M and 10^{-4} M. Both negative (0.9% NaCl) and positive (10^{-6} M N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Sigma Chemical Co., St. Louis, MO) CAS No. 70-25-7) controls were used.

In the sister-chromatid exchange (SCE) test, in order to obtain visible SCEs, 5-bromo-2'-deoxyuridine (BrdUrd, Sigma Chemical Co., St. Louis, MO, final concentration 25 μM) was added to each culture one hour after the beginning of incubation. Two hours before harvesting, colcemide (Ciba, Basel, Switzerland) was added to the cultures to achieve a final concentration of 0.5 μg/ml. After hypotonic treatment (0.075 M KCl) followed by three repetitive cycles of fixation in methanol/acetic acid solution (3 : 1, v/v), centrifugation, and resuspension, the cell suspension was dropped on chilled, grease-free microscopic slides, air-dried, and aged for at least four days. Differential staining for the inspection of the SCE rate was performed according to the fluorescence-plus-Giemsa (FPG) procedure (Perry and Wolff, 1974). A total of 60 well-spread mitoses per donor were scored, and the values obtained were calculated as SCEs per cell.

Finally, in cytokinesis-blocked micronucleus assay, RPMI 1640 (Gibco, Grand Island, NY) was used as a medium, and cytochalasine B (Sigma Chemical Co., St. Louis, MO, final concentration 6 μg/ml) was added 47 h and 30 min after the beginning of incubation. After 72 h the cells were gently rinsed in serum-free RPMI 1640 medium, then exposed to short hypotonic treatment (3 min) with 0.075 M KCl at room temperature. After standard procedure of preparation (three cycles in methanol-acetic acid solution), the staining was performed in 2% Giemsa (Kemika, Zagreb, Croatia) solution in the Gurr buffer (pH = 6.8). At least 1000 binucleated cells per donor were analysed for the presence of micronuclei.

Statistical analysis of experimental values in the SCE test was performed by Student's t-test. In the cytokinesis-blocked micronucleus test, statistical significance was determined by χ^2 test.

Results and Discussion

In this study, the effects of insulin on cultured human peripheral blood lymphocytes were evaluated at the cytogenetic level by monitoring SCE-per-cell frequency and appearance of micronuclei in the cytokinesis-blocked assay. In order to determine changes in the mitotic activity, the mitotic index (MI) was determined for each experimental concentration, as well as for controls.

Experimental values of the MI are presented in Table 1. Insulin significantly increased the percentage of cells in mitosis at concentrations of 0.75×10^{-8} M ($P < 0.01$) and 2.5×10^{-8} M ($P < 0.05$), in comparison to the negative control (MI = 4.73%). At the concentration of 0.75×10^{-8} M, the MI value was increased by 72% as compared to the negative control, and application of insulin in concentration 2.5×10^{-8} M increased MI by 41%. All other concentrations applied in this investigation caused a slight insignificant increase in the MI values. It is noteworthy that maximal mitogenic effect of insulin was obtained at the concentration of approximately 10^{-8} M. In many other studies insulin expressed maximal biological effects at comparable concentrations (the same order of magnitude). Thus, insulin increases malic enzyme gene expression at 0.4×10^{-8} M in rat differentiating brown adipocytes (Garcia-Jimenez et al., 1993), maximally stimulates DNA synthesis in mouse embryonic fibroblastic 3T3-F442A preadipose cells (Tang et al., 1995) at 0.5×10^{-8} M, and rapidly decreases the amount of IGF-1-binding protein mRNA in human HepG2 cells at 10^{-8} M (Babajko, 1995).

Although it has been shown that insulin acts as a mitogen (Ish-Shalom et al.,1995; McIntyre et al., 1998), subcellular events responsible for stimulation of DNA synthesis, activation of protooncogenes and progression through the cell cycle are not completely understood. At present, it is suggested that insulin-mediated stimulation of mitogenesis may result, at least in part, from the well-known growth factor cascade of serine/threonine kinases (Lazar et al., 1994). The mitogen-activated protein kinase (MAP kinase) is regulated after activation of the *ras* protooncogene due to a series of protein-protein interactions initiated by receptor tyrosine kinases (Pelech and Sanghera, 1992). It is possible that insulin-elevated activities of the MAP kinase and ribosomal S6 kinase (Rsk) constitute a step in the kinase pathway that transduces mitogenic signals into the nucleus (Huang and Erikson, 1996).

Biochemical changes following insulin binding to its receptors and subsequent signal transduction have been extensively studied in many laboratories. However, possible genotoxic effects have not been thoroughly investigated. To evaluate such possibilities, we analysed SCE-per-cell frequency and micronuclei in cytokinesis-blocked assay.

The SCE test is considered to be more sensitive than analysis of chromosome aberrations. Although the molecular mechanisms of SCE induction remain unclear, it seems that SCEs reflect DNA breakage and reunion at homologous loci of the sister chromatids (Iannuzzi et al., 1991).

The results of *in vitro* SCE test are shown in Table 1. The average number of SCEs per cell was 6.27 ± 0.29 in the negative control, whereas insulin treatment caused only a slight departure from the control value. An elevated rate of SCE-per-cell frequency ($P < 0.001$)