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Interleukin-1-induced expression of nitric oxide synthase in insulin-producing cells is preceded by c-*fos* induction and depends on gene transcription and protein synthesis

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The cytokine interleukin 1β (IL-1β) induces the expression of an isoform of nitric oxide synthase (NOS) in insulin-producing cells which is similar to that expressed in activated macrophages. In the present study we show that IL-1β-induced expression of NOS mRNA in these cells is preceded by expression of c-*fos* mRNA. Moreover, the stimulatory effects of recombinant IL-1β on NOS mRNA expression are prevented by co-incubation with an inhibitor of gene transcription (actinomycin D) or an inhibitor of protein synthesis (cycloheximide). These data suggest that IL-1β-induced NOS mRNA expression may be mediated by transcription of immediate early response genes, and that c-*fos* may be one of these genes.

Interleukin-1β; Nitric oxide; Nitric oxide synthase; HIT cell; Insulin-producing cell; Insulin-dependent diabetes mellitus

1. INTRODUCTION

Long-term in vitro exposure of rodent pancreatic islets to the cytokine IL-1 (IL-1) induces suppression of insulin release and (pro)insulin biosynthesis, and may lead to β-cell death [1,2]. We and other groups have recently characterized some of the mechanisms behind the effects of IL-1 on rat islets. Thus, the actions of the cytokine requires binding to membrane receptors [3,4], induction of gene transcription and protein translation [5,6]. After an initial stimulation of islet oxidative metabolism and insulin release, there is a progressive functional inhibition [1,2]. This is caused by formation of the reactive compound, nitric oxide (NO) [7,8], which induces an impairment in mitochondrial function and decreases ATP production [2], probably by impairing the function of the Krebs cycle enzyme, aconitase [8].

NO formation is controlled by the enzyme, nitric oxide synthase (NOS). Different tissues present different isoforms of NOS [9], and we have recently shown that IL-1β induces the expression of an isoform of NOS in insulin-producing cells, which is similar to that observed in activated macrophages [10]. Considering that NO generation may be a final common pathway for cytokine(s)- and/or macrophage-induced β-cell damage in insulin-dependent diabetes mellitus (IDDM) [11,12], it is of relevance to further characterize the genetic control of the inducible form of NOS in insulin-producing cells [10]. In the current study, we investigated the time-course of the effects of recombinant IL-1β on NOS and c-*fos* mRNA expression in the clonal insulin secretory cell line, HIT-T15 cells (HIT cells) [13]. Moreover, the effects of blockers of gene transcription (actinomycin D) or blockers of protein synthesis (cycloheximide) on NOS mRNA expression were also evaluated. Finally, we characterized the expression of the mRNAs for the types I and II IL-1 receptors in these cells.

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2. EXPERIMENTAL

2.1. *Materials*

Human recombinant IL-1β (rIL-1β) was a kind gift from Dr. K. Bendtzen, Laboratory of Medical Immunology, Righospitalet, Copenhagen, Denmark. rIL-1β was produced by Immunex Research and Development Corp. (Seattle, WA, USA) and presented a biological activity of 50 U/ng [14]. The chemicals were obtained from the following sources: FastTrack mRNA isolation kit from Invitrogen Corp. (San Diego, CA, USA); Megaprime DNA labelling system and [α-^{32}P]dCTP from Amersham International (Amersham, UK); MagnaGraph nylon transfer membrane from Micron Separations Inc. (Westboro, MA, USA); actinomycin D from Sigma Chemicals (St. Louis, MO, USA); cycloheximide (Calbiochem-Bohering Corp, La Jolla, CA, USA). All other chemicals or analytical grade were obtained from E. Merck (Darmstadt, Germany) or from Sigma Chemicals.

2.2. *Methods*

The clonal hamster insulin secretory cell line, HIT-T 15 (HIT cells) [13] was originally obtained from Dr. S.J. Ashcroft, Oxford, UK. Growing HIT cells were trypsinized and subcultured in RPMI 1640 supplemented with 10% (v/v) FCS, as previously described [10].

Exposure of the HIT cells to rIL-1β (1 ng/ml) was performed for 30 min or 1, 2, 4, 6 and 8 h as described above. HIT cells were exposed to rIL-1β (1 ng/ml) in the presence of actinomycin D (5 μg/ml) or cycloheximide (20 μg/ml) for a 6-h period. The concentration of cycloheximide used (20 μg/ml) decreases the total protein biosynthesis of HIT cell to less than 10% of control values (data not shown). Follow-

ing rIL-1β exposure, aliquots of the culture medium (80 μl; collected in triplicate) were deproteinized and the nitrite content determined as previously described [8].

For Northern blot analysis, poly(A)$^+$-selected mRNA was isolated from 10^7 to 3×10^7 cells using a FastTrack mRNA isolation kit. Following isolation, the mRNA samples (2–4 μg) were electrophoresed on an agarose gel, transferred onto a nylon membrane [15], and sequentially hybridized to ^{32}P-labelled cDNA probes coding either for the inducible form of NOS [16] (a kind gift from Drs. C.R. Lyons, G.J. Orloff and J.M. Cunningham, Hematology-Oncology Division, Harvard Medical School, Boston, MA, USA) or for the viral protooncogene, c-*fos* [17] (a kind gift of Dr. Natalie Teich, Imperial Cancer Research Fund, London, UK). As an internal control, the membranes were re-hybridized to a cDNA probe coding for the human glyceraldehyde- 3-phosphate dehydrogenase gene (GAPDH [18]) (obtained from American Type Culture Collection, Rockville, MD, USA). GAPDH mRNA levels are unaffected by large variations in β-cell functional activity [19] or by different stages of cell growth, in distinct cell lines [20]. In a separate series of experiments, mRNA (5 μg) obtained from control HIT cells, or HIT cells exposed for 6 h to rIL-1β, was hybridized with cDNA probes encoding the murine forms for either type I or type II IL-1 receptors [21,22] (a kind gift of Dr. John Sims, Department of Molecular Biology, Immunex Research and Development Corp.). Hybridization and autoradiography were performed as previously described [15].

Data are presented as means ± S.E.M., and groups of data were compared using paired or unpaired Student's *t*-test as appropriate.

3. RESULTS AND DISCUSSION

The effects of IL-1 on different cell types are exerted via specific receptors for IL-1 [23]. The two better characterized IL-1 receptors are the M_r 80,000 type I receptor, present mostly in T-lymphocytes and fibroblasts, and the M_r 60,000 type II receptor, present mostly in B-lymphocytes, bone marrow cells and hepatoma cells [21–23]. In order to obtain information regarding the type(s) of IL-1 receptor(s) present in insulin-producing cells, mRNA obtained from HIT cells was sequentially hybridized with cDNAs encoding the type I and the type II IL-1 receptors. As shown in Fig. 1, HIT cells expressed a 5.0 kb mRNA for the type I IL-1 receptor, and the mRNA content was not modified by 6 h (Fig. 1) or 24 h (data not shown) exposure to rIL-1β (1 ng/ml). When the blots were re-hybridized with a type II IL-1 receptor probe (Fig. 1), two major bands, estimated as 1.5 and 0.5 kb, were detected. The 1.5 kb band is similar to the type II IL-1 receptor mRNA previously described in different B-lymphocyte cell lines [22]. Indeed, in another experimental series in which mRNA obtained either from HIT cells or from the murine B cell line, M 12.4.1.C 3 (a kind gift from Drs. E. Michaelson and R. Holmdahl, Department of Medical and Physiological Chemistry, Uppsala University, Uppsala, Sweden), were hybridized in parallel with the type II IL-1 receptor probe (data not shown), both HIT cells and murine B cells showed a similar 1.5 kb band, but only HIT cells presented the smaller 0.5 kb band. Interestingly, the larger band seemed to be induced by IL-1 in HIT cells, both after 6 h (Fig. 1) or 24 h (not shown) exposure to the cytokine. Thus, HIT cells present

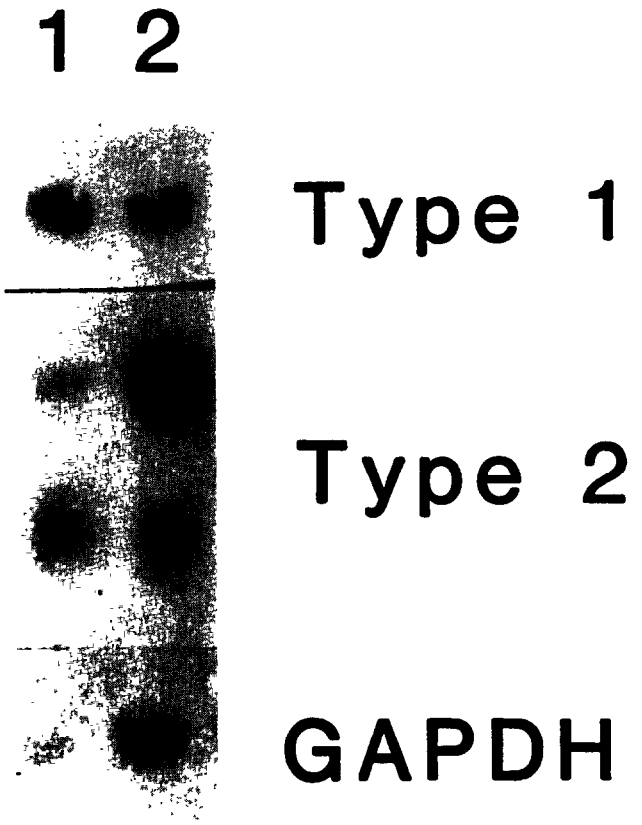

Fig. 1. Northern blots hybridized with a probe for the type I IL-1 receptor (top), with a probe for the type II IL-1 receptor (middle) or a probe for GAPDH (bottom). HIT cells were cultured under control conditions (lane 1) or exposed to rIL-1β for 6 h (lane 2), and then harvested for mRNA extraction. The figure is representative of 2 separate experiments.

mRNAs for both the type I and type II IL-1 receptors. Similar observations were made in some B- and T-lymphocyte cell lines [22]. Previous studies, with co-incubation of insulin-producing cells with IL-1 and/or an human interleukin-1 receptor antagonist protein (IRAP), which binds preferentially to the murine type I IL-1 receptor [24], have shown complete blocking of the suppressive effects of IL-1 by IRAP [4]. Moreover, antibody blocking of the murine type I IL-1 receptor also protected these cells against IL-1 [25]. Given these previous observations, the presence of type II IL-1 receptor mRNA in HIT cells is surprising. It still remains to be clarified if the signalling pathways used by types I and II IL-1 receptors are similar. When available, this information may be of relevance to further explain the different actions of IL-1 on insulin-producing cells [2].

Following binding to surface receptors, IL-1β transduces a signal(s) in insulin-producing cells that leads to increased expression of the inducible form of NOS [10], followed by NO formation [7,8]. In order to further investigate this issue, we performed time–course studies on the actions of rIL-1β on NOS mRNA expres-

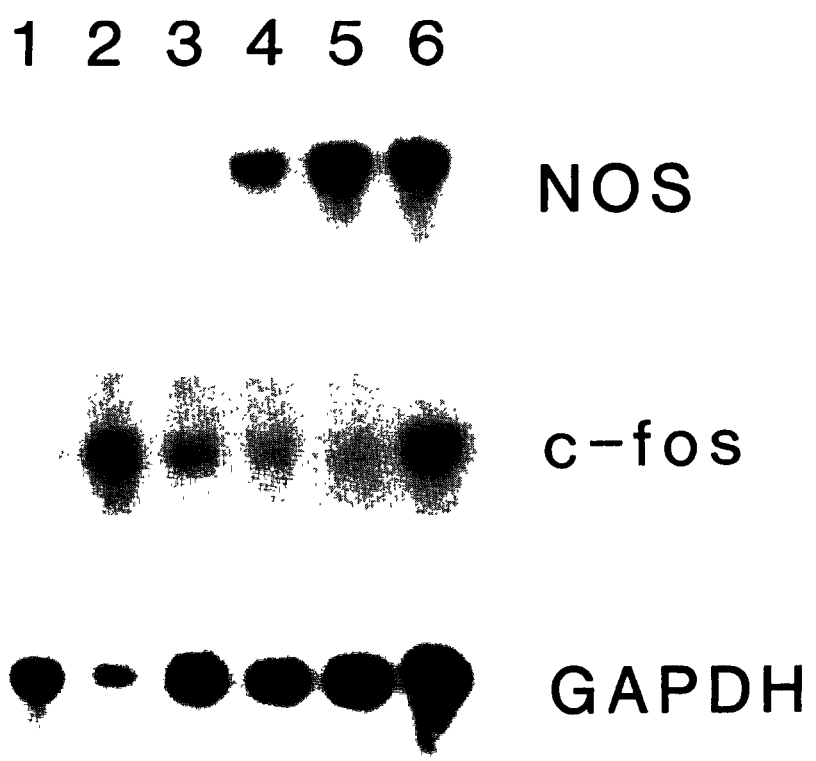

Fig. 2. Northern blot analysis of HIT cells at different time points of culture in the presence of the cytokine rIL-1β (1 ng/ml). Hybridizations were performed with cDNAs encoding for the inducible form of NOS (top), the c-*fos* protooncogene (middle) or GADPH (bottom). Lane 1, control; lane 2, rIL-1β 1h; lane 3, rIL-1β 2 h; lane 4, rIL-1β, 4 h; lane 5, rIL-1β, 6 h; lane 6, rIL-1β, 8 h. The figure is representative of 3 separate experiments.

sion (Fig. 2). After 4 h exposure to the cytokine, there was already a clear induction of a 5.0 kb mRNA, similar to previous observations in activated macrophages [16] and HIT cells [10]. The amounts of this mRNA were further increased after 6 and 8 h (Fig. 2). Parallel determinations of nitrite, an end product of NO, in the culture medium showed a similar pattern as observed with NOS mRNA, i.e. an initial increase in nitrite accumulation after 4 h rIL-1β exposure, with further increases in nitrite content after 6 and 8 h (Fig. 3).

IL-1 induces an early increase c-*fos* mRNA in pancreatic B-cells [26]. The protein product of the c-*fos* gene has been proposed to function as a transcriptional regulator, which couples early signal transduction to late nuclear events [27]. Thus, it is conceivable that the effects of IL-1 in insulin-producing cells are mediated by early transcription of the c-*fos* gene, followed by synthesis of the Fos protein (and/or other early response genes), leading to induction of NOS transcription. In line with this possibility, re-hybridization of the blots (Fig. 2) with a probe for c-*fos* revealed an early and transitory (30 min–1 h; Fig. 2 and data not shown) increase in c-*fos* mRNA contents, preceding by 2–3 h the increase in NOS mRNA. It is noteworthy that after 8 h, coinciding with the observed increase in NOS mRNA and nitrite production, there was a second increase in c-*fos* mRNA content. Besides its role as early

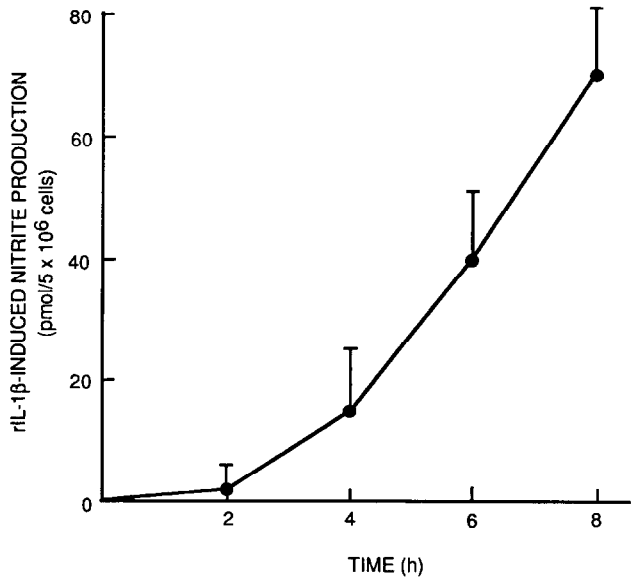

Fig. 3. rIL-1β-induced medium nitrite accumulation in HIT cells exposed for different amounts of time to the cytokine (1 ng/ml), as compared to nitrite accumulation into the medium of HIT cells cultured in the absence of rIL-1β. At each time point, the medium nitrite content observed in control HIT cells was subtracted from the values observed in the respective rIL-1β-treated HIT cells. Results are means ± S.E.M. for 3–4 separate experiments

Fig. 4. Northern blot analysis of HIT cells exposed for 6 h to rIL-1β and/or actinomycin D (5 μg/ml) or cycloheximide (20 μg/ml). Hybridizations were performed with cDNAs encoding for the inducible form of NOS (top) or GADPH (bottom). Lane 1, control; lane 2, cycloheximide; lane 3, actinomycin D; lane 4, rIL-1β; lane 5, rIL-1β + cycloheximide; lane 6, rIL-1β + actinomycin D. The figure is representative of 3 separate experiments.

response gene, c-*fos* can also be induced as a late response to cell damage and/or NO generation [27,28]. Indeed, in separate experiments, where HIT cells were exposed to the alkylating agents, streptozotocin or methyl methanesulfonate (Björklund and Eizirik, unpublished data), c-*fos* mRNA was induced after 3–4 h, and this increase in mRNA lasted for at least 7–9 h. Thus, it is conceivable that, in the current series of experiments, we observed two facets of c-*fos* mRNA induction: an initial and transitory induction of c-*fos* by IL-1, probably playing a role of an early response gene, followed by a second induction of the gene, probably as a consequence of NO-induced cell damage.

The role of transcriptional regulation and protein synthesis for rIL-1β-induced NOS mRNA expression were evaluated in a second series of experiments, using the RNA synthesis inhibitor, actinomycin D, and the protein synthesis inhibitor, cycloheximide (Fig. 4). Both drugs has previously been shown to prevent IL-1-induced NO production [8,29] and to protect β-cells

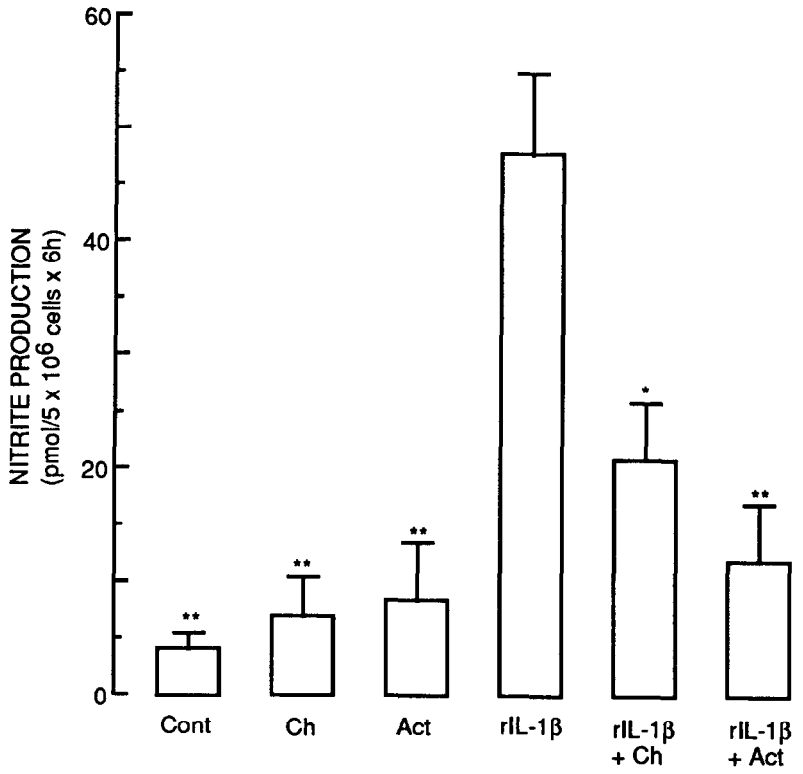

Fig. 5. Inhibition of nitrite accumulation by actinomycin D (Act, 5 μg/ml) or cycloheximide (Ch, 20 μg/ml) in HIT cells exposed for 6 h to rIL-1β (1 ng/ml). Results are means ± S.E.M. for 6 separate experiments. $^{*}P < 0.02$ and $^{**}P < 0.01$ when compared to HIT cells exposed to rIL-1β alone.

against suppressive effects of the cytokine [5,6]. Actinomycin D completely prevented the rIL-1β-induced increase in NOS mRNA (Fig. 4). and nitrite accumulation into the culture medium (Fig. 5). This observation suggests that expression of IL-1-induced NOS mRNA in HIT cells is regulated, at least in part, at the transcriptional level. Cycloheximide also prevented the increase in NOS mRNA and, at least partially, prevented rIL-1β-induced nitrite accumulation into the medium (Figs. 4 and 5). These effects of cycloheximide suggest that synthesis of new proteins is a prerequisite for induction of NOS mRNA. As discussed above, it is conceivable that one of these proteins is the protein encoded by the c-*fos* protooncogene.

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