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INTERLEUKIN-1β INDUCED ACTIVATION OF NF-κB IN INSULIN PRODUCING RINm5F CELLS IS PREVENTED BY THE PROTEASE INHIBITOR Nα-p-TOSYL-L-LYSINE CHLOROMETHYLKETONE

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Summary: The cytokine Interleukin-1β (IL-1β) is known to exert cytotoxic effects upon rodent β-cells in vitro by inducing nitric oxide production and has therefore been suggested to play a role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM). Using the insulin producing rat cell line RINm5F and an electrophoretic mobility shift assay (EMSA), it was presently found that IL-1β induced a rapid activation (5 min) of the transcription factor NF-κB and that this event was prevented by the protease inhibitor Nα-p-tosyl-L-lysine chloromethylketone (TLCK). TLCK prevented also IL-1β induced nitric oxide production. It is concluded that NF-κB activation may be a necessary signal for IL-1β induced β-cell damage and that this process can be modulated by specific protease and NF-κB inhibitors.

It has been demonstrated that IL-1β exerts inhibitory and cytotoxic effects on rodent pancreatic β-cells in vitro (1-3) and that this effect is probably mediated by the induction of the enzyme nitric oxide synthase (iNOS) (4). Nitric oxide is a short-lived and reactive radical, which inhibits the Krebs-cycle enzyme aconitase in β-cells leading to decreased glucose oxidation rates, ATP generation and insulin production (2-6). The intracellular signals generated in β-cells by the interaction between IL-1β and its receptor have not been elucidated yet. We have in a previous study not been able to observe any early effects of IL-1β on protein kinase C activity and Ca^{2+}, cAMP and IP_3 levels in isolated

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rat islets (7). However, an early and transient c-fos mRNA induction in response to IL-1β was observed in the rat islet cells and in the insulin producing cell line HIT-T15 (8,9) and the use of inhibitors of tyrosine kinases has indicated that tyrosine phosphorylation events may mediate the IL-1β signal (10,11).

The transcription factor NF-κB is present in most cells and is thought to act as an early immune and inflammatory response mediator (12). It is known to be activated by a multitude of factors such as viruses, bacteria, oxidative stress and cytokines, and to induce the transcription of genes encoding defence and signalling proteins. NF-κB is sequestered in the cytosol of non-stimulated cells as an inactive trimer consisting of p50, Rel-A (p65) and IκB. Upon activation, IκB is released from p50 and Rel-A leading to translocation of the p50/Rel-A dimer to the nucleus where it binds to the B motif; a decameric DNA sequence motif originally observed in the intronic κ light chain enhancer in B-cells (13). Recently, it was reported that rapid proteolysis of IκB is necessary for activation of NF-κB, and that this process is inhibited by serine protease inhibitors such as tosyl-lysine chloromethylketone (TLCK) (14). We have previously observed that TLCK counteracts IL-1β induced nitric oxide production and inhibition of insulin release (15-17). The aim of this study was therefore to study whether IL-1β activates NF-κB in insulin producing cells and in that case, whether this process can be modulated by TLCK.

Materials and Methods

Chemicals Human recombinant IL-1β was kindly provided by Dr. K. Bendtzen, Laboratory of Medical Immunology, Rigshospitalet, Copenhagen, Denmark. The cytokine was produced by Immunex (Seattle, WA) and had a biological activity of 50 U/ng, as compared with an interim international standard rIL-1β preparation (NIBSC, London, UK) (18).

Subcellular fractionation When reaching 50-80% confluency, the clonal insulin-secretory rat cell line RINm5F (5-10 x 10^6 cells) was incubated with IL-1β (50 U/ml) and TLCK (100 μM). Following IL-1β exposure, cells were washed with cold PBS, harvested and resuspended in 100 μl of buffer A (19). After 10 min, cells were pelleted, resuspended in 100 μl of the same buffer and mechanically homogenized. Nuclei were pelleted (20 s at 12000 g) and briefly sonicated in 100 μl of buffer C. After sonication, the nuclear samples were allowed to stand for 30 min at 4 °C. After a brief centrifugation, the remaining supernatants were added to 2 ml of buffer D and concentrated by centrifugation in Centricon microconcentrators (Amicon, Beverly, MA, USA). Meanwhile, to the cytosolic fractions was added 0.11 volumes of buffer B. The cytosolic fractions were then centrifuged at 12000 g for 30 min and the resulting supernatants

were diluted into 2 ml of buffer D as were the nuclear fractions and concentrated likewise. Protein concentrations of the nuclear and cytosolic fractions were determined using the Bradford reagent.

EMSA For electrophoretic mobility shift assays (EMSA), a double-stranded kB 26 mer oligonucleotide (5'-AGCTTCAGAGGGGACTTTCCGAGAGG) (20) was used. As a negative control, a mutated 27 mer was used (5'-AGCTTCAGAAATTAACTTTCC GAGAGG). The double standed oligonucleotides were labelled with [^{32}P]dCTP using Megaprime labelling kit (Amersham) and extracted once with an equal volume of phenol/chloroform/isoamylalcohol (25/25/1, v/v). Equal amounts of cytosolic and nuclear protein (5-10 µg) were denatured in 27% formamide prior to incubation with oligonucleotide (21). Binding reactions contained 10 mM Tris, pH 7.5, 0.2 % deoxycholic acid, 40 mM NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 2 µg polydeoxyinosinic-deoxycytidylic acid and 0.1 ng DNA (14000 cpm). Each 20 µl reaction was incubated at room temperature for 30 min. Samples were separated on 5% non-denaturing polyacrylamide gels in 0.5 x TBE. Band intensities were quantified by densitometric scanning and data are expressed as means ± S.E.M, and groups were compared using Student's paired t-test.

Nitrite determination RINm5F cells (5 x 10^5) were incubated for 6 h with or without IL-1β (50 U/ml) and TLCK (20, 50 100 and 200 µM). Medium samples (80 µl) were then taken and nitrite production was determined as previously described (6).

Results and Discussion

Activation of the transcription factor NF-κB is characterized by translocation of the p50/Rel-A dimer from the cytosol to the nucleus, a process which can be studied by determining the distribution of NF-κB activtity, as assessed by electrophoretic mobility shift analysis (EMSA), between the two subcellular fractions. In the present experiments, both nuclei and cytosol were pretreated with formamide and the reactions were performed in the presence of deoxycholic acid, treatments that prevent IκB binding to and inhibition of p50/Rel-A dimer activity (21). Thus, signals presently obtained represent total NF-κB activity; both activated and IκB-inactivated. Using this experimental approach, we presently observed that a 5 min IL-1β exposure induced a three-fold increase in nuclear NF-κB ($p<0.05$, Student's t-test), and that a 10 min exposure period induced a 6-fold increase ($p<0.01$) (Fig. 1). No effect could be demonstrated after only 2 min of IL-1β exposure (Fig. 1). In a separate series of experiments, we also observed that IL-1β effect persisted for at least 120 min after cytokine addition (Fig. 2).

Nitrite production from control RINm5F cells was 2.6 ± 1.7 and from IL-1β (50 U/ml) incubated cells 40.2 ± 8.2 pmol/5 x 10^5 cells and 6 h ($p<0.05$, Student's t-test, n=3). Nitrite production from cells incubated with IL-1β and 20, 50, 100 and 200 µM of TLCK

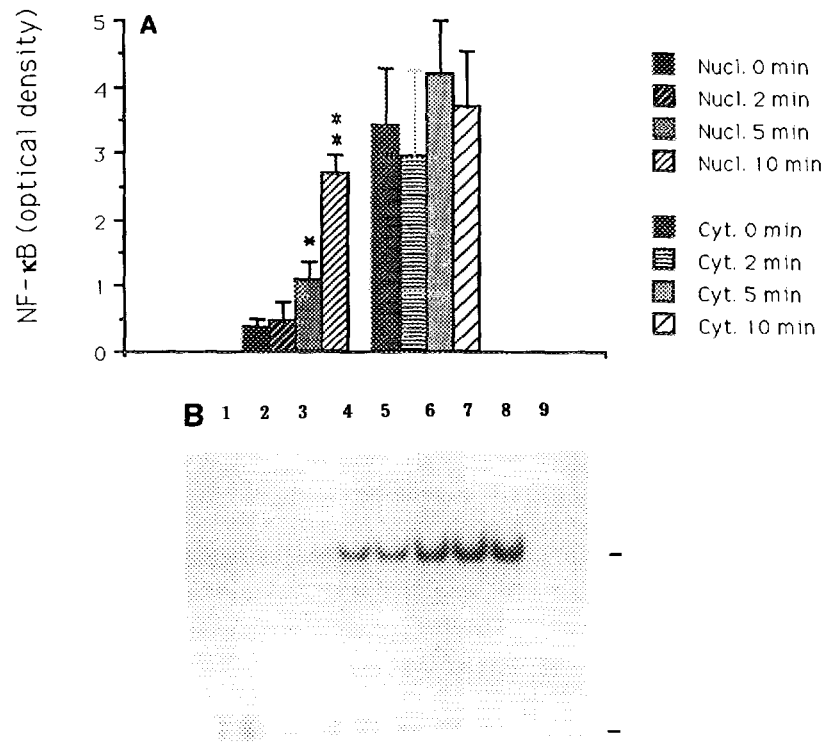

Figure 1.
Effects of IL-1β exposure on subcellular distribution of NF-κB activity in RINm5F cells as determined by EMSA. Panel A: RINm5F cells were exposed to IL-1β (50 U/ml) for 0, 2, 5 and 10 min and then harvested for subcellular fractionation and EMSA using a [^{32}P]dCTP labelled 26 mer (20). NF-κB bands were quantified using densitometry. Results are expressed as means ± S.E.M. for 3-4 separate experiments. *And ** denote $p<0.05$ and $p<0.01$, respectively, vs control using Student's paired t-test. Panel B: Autoradiograph showing a typical EMSA experiment. Lanes 1-4: nuclear fractions. Lanes 5-8: cytosolic fractions. Lane 9: negative control using mutated oligonucleotide. Lanes 1 and 5: control; lanes 2 and 6: 2 min; lanes 3 and 7: 5 min; lanes 4 and 8: 10 min of IL-1β exposure. Positions of retarded oligonucleotide (top) and free oligonucleotide (bottom) are indicated on the right.

was 6.5 ± 3.2, 2.3 ± 1.9, 2.0 ± 1.0 and 1.7 ± 1.7 pmol/5 x 10^5 and 6 h, respectively ($p>0.05$, n=3). Thus, TLCK prevented IL-1β induced nitrite production already at 20 μM. We have previously found that the serine protease inhibitor TLCK protected against IL-1β induced nitric oxide production and inhibition of insulin release in rat pancreatic islets, and that this effect occurs early in IL-1β induced signal transduction (15-17). Moreover, it has been demonstrated that TLCK prevents IL-1β induced NF-κB activation in pre-B cells by inhibiting proteolysis of IκB (14). We presently observed that 100 μM of TLCK

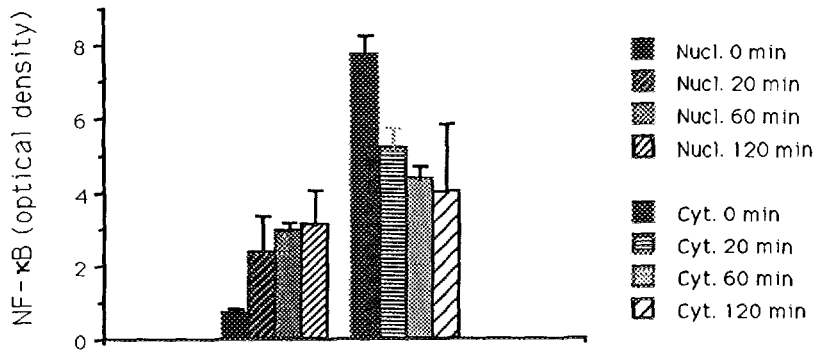

Figure 2.

Effects of IL-1β exposure on subcellular distribution of NF-κB activity in RINm5F cells as determined by EMSA. RINm5F cells were exposed to IL-1β (50 U/ml) for 0, 20, 60 and 120 min. Results are expressed as means ± S.E.M. for 2-3 separate experiments.

prevented IL-1β induced translocation of NF-κB to the nuclei of RINm5F cells (Fig. 3). In view of this finding and that a binding site for NF-κB in the mouse iNOS gene enhancer region has been reported (22), we propose that activation of NF-κB may be a direct and necessary step in IL-1β induced signal transduction leading to iNOS gene transcription in rat β-cells. That TLCK would act at levels prior to NF-κB activation does not seem likely, since TLCK did not interact directly with IL-1β (17), nor do the protease inhibitors used in study by Henkel et al., (14) seem to interact with IL-1 receptor signalling.

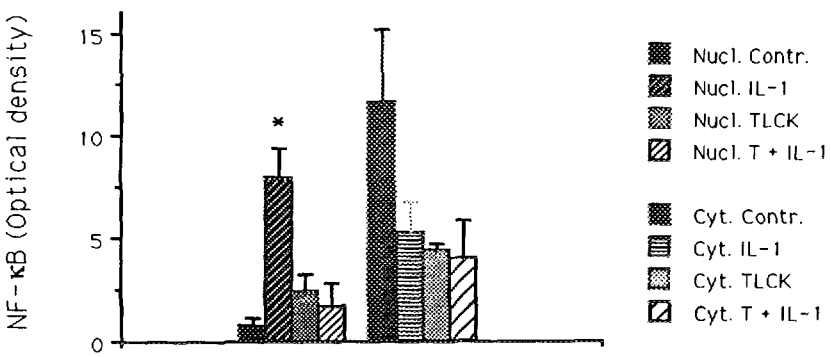

Figure 3.

Effects of TLCK and IL-1β on subcellular distribution of NF-κB activity in RINm5F cells as determined by EMSA. RINm5F cells were exposed to IL-1β (50 U/ml) for 10 min and TLCK (100 μM), which was added 15 min before IL-1β, and then harvested for EMSA. Results are expressed as means ± S.E.M. for 3-4 separate experiments. * Denotes $p<0.05$ vs control using the Student paired t-test.

The mechanism by which the putative TLCK-sensitive protease activates NF-κB in insulin producing cells is unknown. In other cells, it is thought that IκB is released from NF-κB by induction of reactive oxygen intermediates and/or phosphorylation events and that this renders IκB sensitive to proteolytical degradation (12,23). Previous studies, however, have failed to demonstrate a protective action by oxygen radical scavengers against IL-1β induced β-cell supression (3,24-26). Instead, we and others have recently observed that phosphorylation events, both on serine/threonine and tyrosine residues, may mediate IL-1β induced signal transduction (10,11).

Exposure of rat islet cells to IL-1β has been shown to induce not only iNOS, but also heat shock protein 70 (hsp70), heme oxygenase and Mn-superoxide dismutase (Mn-SOD) (27,28). These are all stress response proteins known to be induced by cytokines, heat shock and oxidative stress (29,30), and it could be conceived that induction of these genes also requires NF-κB/Rel activation, either by direct trans-activation or indirectly by interaction with some other transcription factor. Indeed, a NF-κB site has recently been demonstrated in the human heme oxygenase gene (31). Interestingly, it has been recently observed that although NF-κB/Rel binding to the mouse iNOS promoter region is direct and cycloheximide insensitive, the subsequent binding of other transcription control proteins is cycloheximide senstitive (32). Thus, newly synthesized proteins are required to interact with NF-κB/Rel in activating iNOS gene expression (32). This would explain the time lag between addition of IL-1β and the expression of iNOS, hsp70, heme oxygenase and Mn-SOD, which appears to be the same for the different genes, i.e. 2-3 hours (15,16,25,27,28).

In summary, IL-1β induction of NF-κB in insulin producing cells appears to be an important event leading to the expression iNOS. As already proposed by others (14), specific inhibitors of IκB proteolysis may prove useful as general anti-inflammatory and immunosuppressive agents, and assuming that nitric oxide generation plays a deleterious role in insulin-dependent diabetes mellitus, they might also be of use in the prevention of β-cell destruction.

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