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Tetrahydrobiopterin Synthesis Precedes Nitric Oxide-Dependent Inhibition of Insulin Secretion in INS-1 Rat Pancreatic β-Cells

R. Laffranchi,* G. Schoedon,† N. Blau,‡ and G. A. Spinas*

**Division of Endocrinology and Diabetology, Department of Internal Medicine, University Hospital, CH-8091 Zürich, Switzerland; †Department of Medicine, University Hospital, CH-8091 Zürich, Switzerland; and ‡Division of Clinical Chemistry and Biochemistry, Department of Pediatrics, University Hospital, CH-8091 Zürich, Switzerland*

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Interleukin 1 (IL-1) induces pancreatic β-cell dysfunction mainly due to overproduction of nitric oxide (NO). Since tetrahydrobiopterin (BH_4) is a obligatory cofactor of NO synthases, we examined the temporal relationship of BH_4 synthesis, NO production and insulin secretion in a pancreatic β-cell line (INS-1) which was exposed to IL-1. IL-1 affected BH_4 synthesis in a time- and concentration-dependent manner. At a concentration of 10ng/ml IL-1 caused an increase in intracellular BH_4 with peak levels being observed at 6 hours followed by a steady decline in the cellular BH_4 content. The increase in BH_4 synthesis was followed by enhanced NO production and, consecutively, inhibition of insulin secretion. The concentration-dependent regulation of BH_4 synthesis, NO production and suppression of insulin secretion indicate a functional link between these parameters in pancreatic β-cells.

There is substantial evidence that cytokines such as interleukin-1 (IL-1), tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ) are involved in β-cell destruction and may therefore play a role in the pathogenesis of type I diabetes mellitus (1). In particular, the macrophage product IL-1 is considered to be a main effector molecule mediating both dysfunction and destruction of the β-cell (2). Incubation of pancreatic islets with IL-1 results in a sustained inhibition of insulin secretion, depending on the concentration of the cytokine, the duration of exposure and the functional state of the β-cell (3, 4). The signal cascade of IL-1's action is not completely understood. In rat islets, IL-1 stimulates the expression of the inducible isoform of nitric oxide synthase (iNOS) in the β-cell (5, 6). Nitric oxide (NO) thereby produced impairs β-cell function through binding to iron-sulphur-containing enzymes, *e.g.*, aconitase (7, 8) resulting in cellular ATP depletion and, eventually, cell death.

Tetrahydrobiopterin (BH_4) is an essential cofactor of NOS (9, 10) and is required for dimerization of iNOS subunits to the active enzyme (11, 12). An increase in BH_4 synthesis precedes NO formation in endothelial (13) and glial cells (14).

Since both the insulin-inhibitory and the cytotoxic effect of IL-1 on β-cells can at least partly be ascribed to a suppressive action of NO, and because the availability of BH_4 is absolutely necessary for the synthesis of NO, we investigated kinetics and temporal relationship of BH_4 synthesis, NO formation and insulin secretion in β-cells. INS-1 cells, a rat insulinoma cell line which responds to physiological glucose stimuli, were exposed to IL-1, and the intracellular BH_4 levels, NO production and insulin secretion were measured.

MATERIALS AND METHODS

Materials. RPMI-1640 cell culture medium and other cell culture reagents were from Gibco (Basel, CH). Rat insulin was from Novo Nordisk (Bagsvaerd, DK). Recombinant human IL-1β was purchased from PeproTech (Rocky Hill, NJ). Monomethyl-L-arginine (L-NMMA) was from Alexis (Läufelfingen, CH). All other chemicals were of the highest purity commercially available.

Cell culture. INS-1 cells (passages 80-105) were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (15). Cells were seeded at a density of 5×10^6/10cm dish and precultured for 3 days. Thereafter, medium was changed and IL-1 added.

Insulin and nitrite determination. Insulin accumulation in the medium was determined by a competitive ELISA technique (16, 17) using rat insulin as standard.

Nitrite production was assessed by mixing 100μl of medium of seeded cells with 100μl of Griess reagent (0.1% naphtyl ethylenediamine and 1% sulfanilamide in 5% orthophosphoric acid, 1:1 v/v). The developed colour was determined by measuring the absorbance at 550nm with a Biorad 3550 microplate reader. Concentrations were calculated using $NaNO_2$ as standard.

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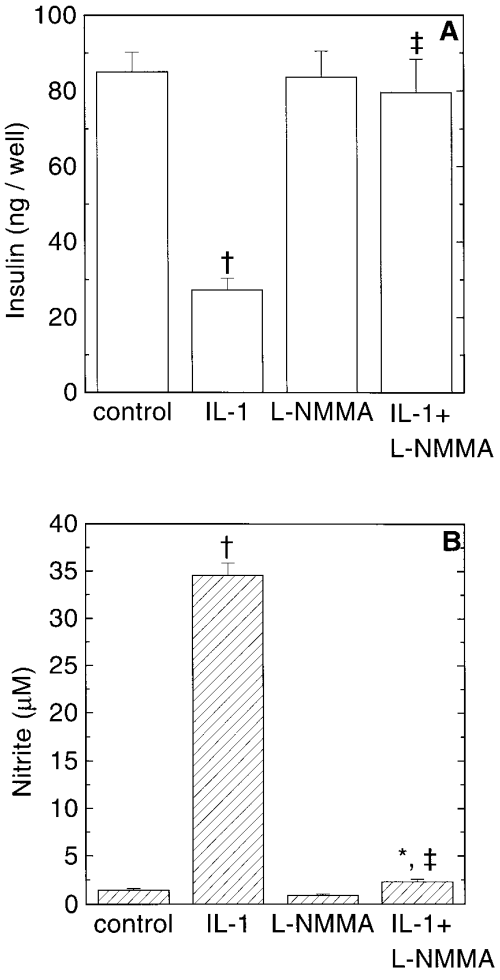

FIG. 1. Effect of IL-1 and L-NMMA on insulin secretion and NO production in INS-1 cells. INS-1 cells were incubated for 24h in the presence or absence of 10ng/ml IL-1 and/or 0.5mM L-NMMA and insulin accumulation (A) and NO formation (B) were measured. Data are mean±SEM of 12 experiments. †, $p<0.001$; *, $p<0.01$ all vs. control. ‡, $p<0.001$ vs. IL-1.

Determination of intracellular BH_4 levels. At the indicated time points, plates were washed 3 times with Geys balanced salt solution and cells were scraped in 500μl of 0.1M HCl. After 3 cycles of freezing/thawing and centrifugation for removal of cell debris, the cytosolic supernatant was oxidized with MnO_2 for 5min, and then deproteinized by filtration through Ultrafree MC microfilters (Millipore) with 10kDa cut off. With this analysis cytosolic BH_4 which is not bound to iNOS is determined. The ultrafiltrate was analyzed for total biopterin (the stable product of BH_4) by HPLC with fluorometric detection as described (18).

Statistical analysis. Statistical differences between means were calculated with the paired Student's t test. $P<0.05$ was considered significant.

RESULTS

Exposure of INS-1 cells for 24h to 10ng/ml IL-1 resulted in a 68% inhibition of insulin release and a 23.5-fold increase in NO production (Fig. 1). Co-incubation of the cells with the NOS inhibitor L-NMMA (0.5mM), which completely abolished NO formation, prevented the IL-1-induced inhibition of insulin release (Fig. 1).

INS-1 cells constitutively synthesize BH_4 (137.9±6.1 pmol/10^7 cells) and NO (1±0.2μM). Interestingly, BH_4 is not released into the culture supernatants (not shown). Stimulation of the cells with 10ng/ml IL-1 increased intracellular BH_4 levels further after 3 and 6h of incubation (ΔBH_4 after 3h: +2±3pmol/10^7 cells; ΔBH_4 after 6h: +14.8±4.0pmol/10^7 cells, n=6, $p<0.05$) (Fig. 2A). Prolonged exposure to IL-1 resulted in a decrease of the cellular BH_4 content (ΔBH_4 after 9h: −28.8±27.2pmol/10^7 cells, after 12h: −71.9±28.6pmol/10^7 cells and after 24h: −50.9±8.8pmol/10^7 cells) (Fig. 2A). The decrease of intracellular BH_4 levels after 6h was accompanied by a continuous increase in NO pro-

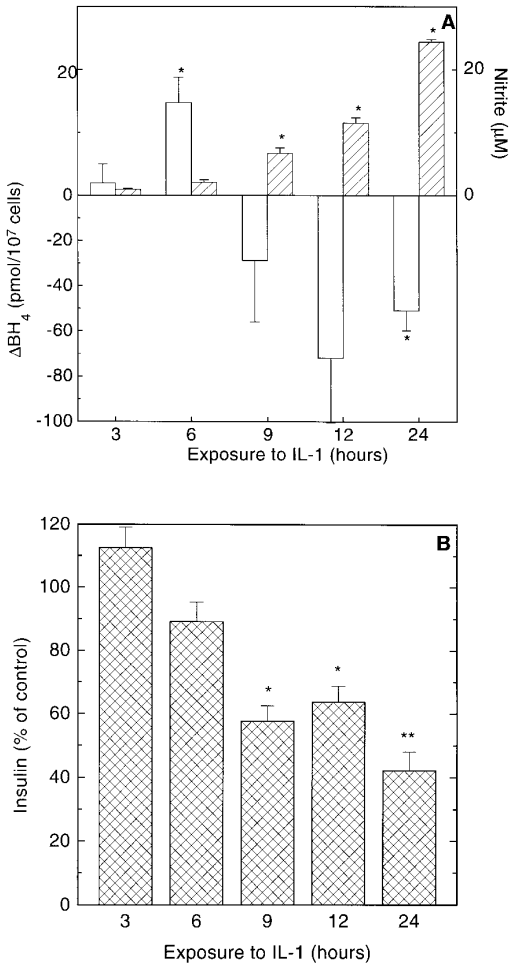

FIG. 2. Time course of intracellular BH_4 synthesis, NO production and insulin release in INS-1 cells exposed to IL-1. INS-1 cells were exposed to 10ng/ml of IL-1 for 3-24 hours. Open bars denote BH_4 and hatched bars nitrite levels (A) and cross-hatched bars insulin release (B). Data are mean±SEM of 3-6 experiments. *, $p<0.05$; **, $p<0.01$ all vs. control.

duction over 24h (6.7-fold after 9h, 11.5-fold after 12h and 24.9-fold after 24h) (Fig. 2A) and a time-dependent decrease of insulin release with maximal inhibition (57.7%) being seen after 24h (Fig. 2B).

As depicted in Fig. 3, addition of IL-1 for 6h resulted in concentration-dependent changes in cellular BH_4 levels, NO synthesis and insulin release. All 3 concentrations of IL-1 (0.1, 1 and 10ng/ml) caused an increase in the BH_4 levels (Fig. 3A): 0.1ng/ml IL-1: $+16.7\pm10.7$pmol/10^7 cells; 1ng/ml IL-1: $+21.5\pm8.0$pmol/10^7 cells; and 10ng/ml IL-1: $+14.8\pm4.0$pmol/10^7 cells. Significant increases (3-fold and 4.2-fold) in NO production were achieved with 1 and 10ng/ml (Fig. 3B), accompanied by inhibition of insulin secretion by 14.2% and 16.9%, respectively (Fig. 3C).

Treatment of the cells for 24h with different concentrations of IL-1 caused a biphasic effect on free BH_4 levels (Fig. 4A). IL-1 at a concentration of 0.1ng/ml augmented the free BH_4 pool by $+19.8\pm7.8$pmol/10^7 cells, while higher IL-1 concentrations (1 and 10ng/ml) resulted in a reduction of the free BH_4 pool by -36.8 ± 24.9pmol/10^7 cells and -50.9 ± 8.8pmol/10^7 cells, respectively. These changes in BH_4 were paralleled by dose-dependent increases in NO formation (Fig. 4B): 5.8-fold with 0.1ng/ml IL-1, 21.9-fold with 1ng/ml IL-1 and 24.4-fold with 10ng/ml IL-1. The increments in NO production resulted in a dose-dependent inhibition of insulin release (Fig. 4C): 10.4% at 0.1ng/ml IL-1, 39.8% at 1ng/ml IL-1 and 52.7% at 10ng/ml IL-1.

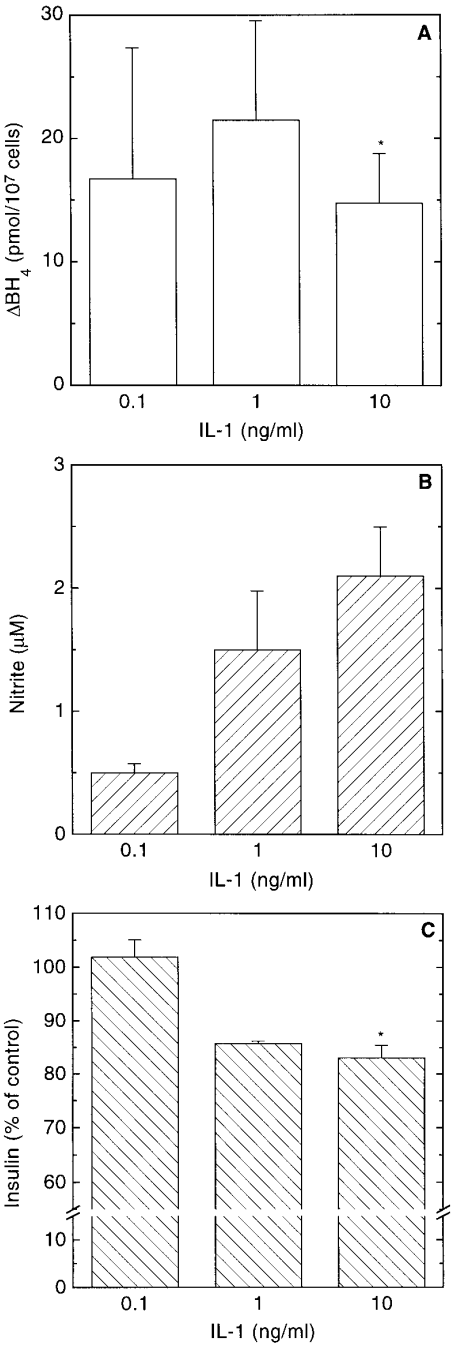

FIG. 3. Concentration-dependent effects of IL-1 after 6h exposure on BH_4 levels, NO production and insulin release in INS-1 cells. INS-1 cells were treated for 6h with 0.1, 1 and 10 ng/ml IL-1 and BH_4 levels (A), NO formation (B) and insulin accumulation (C) were measured. Data are mean±SEM of 3 experiments. *, $p<0.05$ vs. control.

DISCUSSION

It is well established that BH_4 regulates the activity of NOS (19-21). Furthermore, in cells expressing iNOS upon exposure to inflammatory stimuli, BH_4 synthesis is induced by the same stimuli within these cells (22-24). Particularly in the vascular system, a link between BH_4 synthesis and cellular functions has emerged (25-27).

The present study describes for the first time the functionally linked BH_4- NO pathways in the insulin-secreting pancreatic β-cell. The rat insulinoma cells INS-1 constitutively synthesize high levels of BH_4 which are susceptible to modulation by IL-1 (Fig. 2, 3, 4). INS-1 cells did not secrete BH_4 into the medium, even after exposure to IL-1, a feature only found in vascular smooth muscle cells (28, 29). However, IL-1-induced NO production by INS-1 cells was preceded by enhanced BH_4 synthesis. These results are in good agreement with previously published data showing that NO production depends on preceding BH_4 synthesis in endothelial (13) and glial cells (14). The biphasic kinetics of BH_4 (increase after 3 and 6h, decrease after 9, 12 and 24h) indicate that the formed BH_4 is bound by the newly synthesized iNOS protein which needs BH_4 for dimerization and activation (11) (Fig. 2). Maximal NO synthesis occurs only after saturation of the enzyme complex with the BH_4 cofactor, which has to be synthesized in advance (Fig. 3, 4).

Inhibition of insulin secretion occurred exclusively

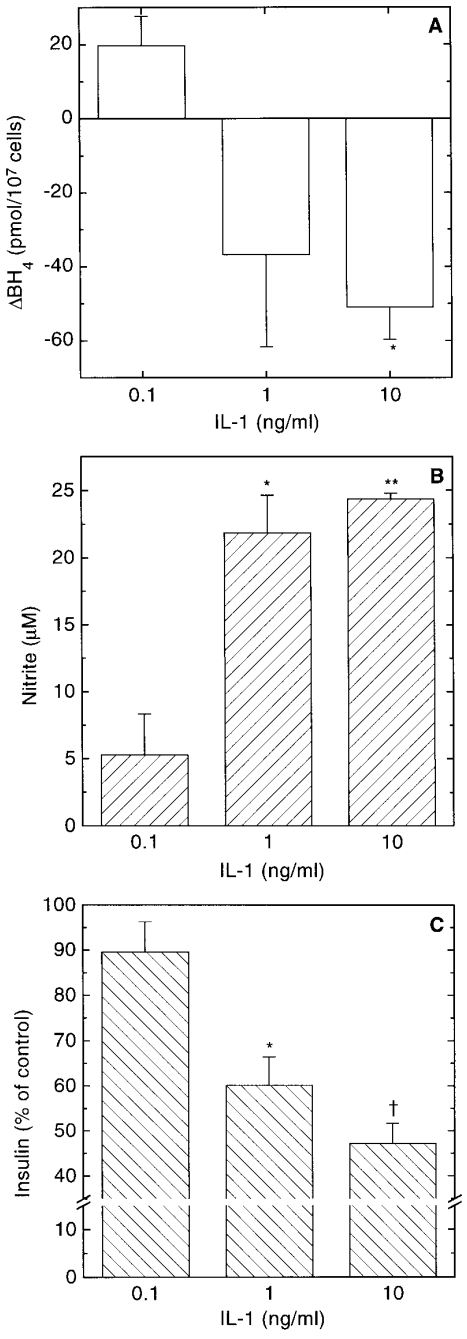

FIG. 4. Concentration-dependent effects of IL-1 after 24h exposure on BH_4 levels, NO production and insulin release in INS-1 cells. INS-1 cells were treated for 24h with 0.1, 1 and 10ng/ml IL-1 and BH_4 levels (A), NO formation (B) and insulin accumulation (C) were measured. Data are mean±SEM of 3 experiments. *, $p<0.05$; **, $p<0.001$; †, $p<0.01$ all vs. control.

after the beginning of NO formation (Fig. 2,4), which is in line with the observation that inhibition of insulin secretion by IL-1 depends on the generation of NO and is prevented by blocking the NOS (Fig. 1). The present findings in INS-1 cells are in agreement with previously published data in rat islets (5, 6, 30). Taken together our data provide evidence for the existence of a functional link between BH_4 synthesis, NO formation, and insulin secretion in pancreatic β-cells.

Further studies are needed to confirm this link using purified β-cells as well as whole islets. Our data suggest that inhibition of BH_4 synthesis could be a reliable approach for suppression of NO overproduction in pathological states, *e.g.*, destruction of β-cells.

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