Regulation of inducible nitric oxide synthase expression in β cells by environmental factors: heavy metals

Walther ECKHARDT, Kerstin BELLMANN and Hubert KOLB[1]

Diabetes Research Institute at the University of Düsseldorf, Auf'm Hennekamp 65, D-40225 Düsseldorf, Germany

The expression of inducible NO synthase (iNOS) in pancreatic islet β cells modulates endocrine cell functions and, at very high levels of NO production causes β-cell death. We tested the hypothesis that environmental factors such as heavy-metal salts modulate iNOS expression in β cells. A rat β-cell line (insulinoma RINm5F) was cultured in the presence of low-dose interleukin (IL)-1β for suboptimal induction of iNOS. $PbCl_2$ (0.1–10 μM) dose-dependently increased NO (measured as nitrite) formation ($P < 0.001$). In contrast, $HgCl_2$ suppressed nitrite production (0.1–10 μM, $P < 0.05$). Measurements of iNOS activity by determining citrulline levels confirmed the potentiating effect of $PbCl_2$ ($P < 0.05$). There was a narrow time window of heavy-metal actions, ranging from -24 h (Hg^{2+}) or -3 h (Pb^{2+}) to $+2$ h, relative to the addition of IL-1β. By semi-quantitative reverse transcriptase-PCR, enhanced levels of iNOS mRNA were found in the presence of Pb^{2+} ($P < 0.05$) and decreased levels in the presence of Hg^{2+}. The amount of iNOS protein as determined by Western blotting was increased in the presence of Pb^{2+}. We conclude that Pb^{2+} upregulates and Hg^{2+} suppresses iNOS gene expression at the level of transcription, probably by acting on the signalling pathway. These observations may have important implications for understanding pathological effects of environmental factors on endocrine organ functions.

Key words: L-citrulline, insulinoma cells, lead, mercury, nitric oxide.

INTRODUCTION

Many cell types respond to inflammatory stress with the expression of inducible NO synthase (iNOS, NOS II). Potent stimulatory agents are of microbial origin, such as LPS or DNA containing non-methylated CpG motifs [1,2]. This fits with iNOS representing a high-output path of NO production for immune defence. Other stimulators of iNOS expression are of endogenous origin. Several cytokines, such as interleukin (IL)-1β and interferon γ (IFNγ), have been reported to induce iNOS in many cell types, whereas IL-10 and transforming growth factor β belong to the group of cytokines suppressing iNOS expression [3–7].

More recently, it has been observed that NO from iNOS is not only a mediator of immune defence but acts upon iNOS-producing cells and other cells in a regulatory fashion, modulating gene transcription, translation and protein function [8–10]. As a consequence, iNOS must be regarded as part of the paracrine regulatory network controlling tissue function. It therefore is conceivable that the modulation of tissue function by environmental factors also occurs via modulating the expression of iNOS. One such tissue in which NO has been described to contribute to physiology and pathology is the pancreatic islet. Induction of iNOS in islet β cells by IL-1β has been reported to potentiate or downregulate insulin production, depending on the strength of the stimulus [11]. In addition, the induction of iNOS in β cells has been suggested to be a major pathway of cell death in chronic inflammation preceding type-1 (insulin-dependent) diabetes [12,13].

We selected heavy-metal salts as candidate environmental factors for modulation of iNOS expression. First, a previous epidemiological study linked the incidence of type-1 diabetes in Sweden to exposure to mercury [14]. Secondly, lead and mercury salts have been observed to modulate NO formation in activated macrophages [15,16]. Whether the latter was due to an action of the heavy-metal salts on iNOS gene transcription, translation or enzyme function in macrophages has not been established.

In the present paper we demonstrate a strong modulatory effect of Pb^{2+} and Hg^{2+} on iNOS expression in an endocrine cell line at the level of gene expression.

MATERIALS AND METHODS

Cell culture

RIN, clone 5F (RINm5F) is a β-cell line derived from an insulinoma of the NEDH rat [17] islet cell tumour. RIN cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C in RPMI-1640 medium (Gibco-BRL, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal-calf serum (Gibco-BRL)/25 mg/l ampicillin/120 mg/ml penicillin/270 mg/l streptomycin (Serva GmbH, Heidelberg, Germany)/1 mmol/l sodium pyruvate/2 mmol/l L-glutamine/10 mmol/l 100× non-essential amino acids (Gibco-BRL)/2 g/l $NaHCO_3$/2.38 g/l Hepes (pH 7.3).

RIN cell culture and iNOS induction

RIN cells were seeded (10^5 cells/200 μl) in 96-well tissue culture plates and allowed to adhere overnight. On the next day, the toxins were added 3 h ('-3 h') before cytokine stimulation for 6–24 h. Afterwards the supernatants were collected for determination of nitrite and L-citrulline content. In a second set of experiments the previous schedule was modified by varying the time points of toxin addition from '-3 h' to '-24 h', '$+2$ h' and '$+6$ h'.

Abbreviations used: iNOS, inducible NO synthase; LPS, lipopolysaccharide; IL, interleukin; IFNγ, interferon γ; NF-κB, nuclear factor κB; PKC, protein kinase C.

[1] To whom correspondence should be addressed (e-mail Kolb@dfi.uni-duesseldorf.de).

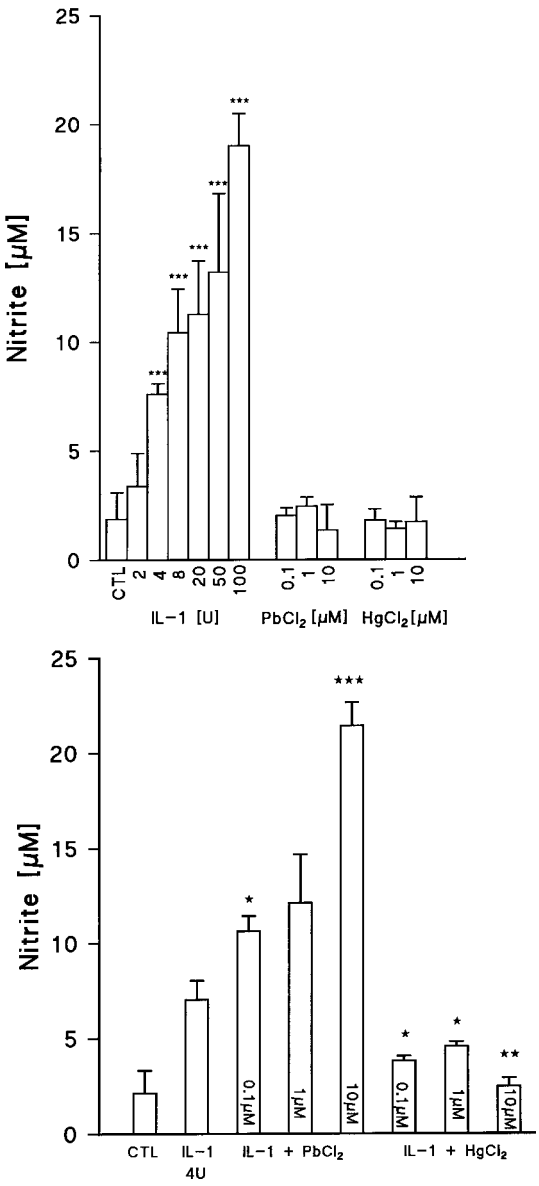

Figure 1 Modulation of nitrite release from RIN cells by IL-1β and heavy metals

(Top panel) Cells (0.5×10^6/ml) were stimulated with the indicated doses of IL-1β [units (U)/ml] or $PbCl_2$/$HgCl_2$ for 24 h or sham-treated (CTL). Shown are means $\pm$ S.D. of at least six experiments. ***, $P < 0.001$ compared with untreated control cells. (Bottom panel) Effect of heavy metals on nitrite release from IL-1β-stimulated RIN cells. The cells were sham-treated (CTL) or stimulated with 4 units (U)/ml IL-1β for 24 h, or 10 μM $PbCl_2$ or 1 μM $HgCl_2$ was added 3 h prior to IL-1β. Shown are means $\pm$ S.D. of at least six experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with 4 units/ml IL-1β.

For the detection of iNOS protein or mRNA, RIN cells were seeded (0.5×10^6 cells/ml) in 10 ml culture dishes and treated following the standard '-3 h' protocol. $PbCl_2$ (Fluka Chemika, Buchs, Switzerland) and $HgCl_2$ (Sigma, St.Louis, MO, U.S.A.) were dissolved prior to each experiment in PBS at 1 mM and diluted with medium.

Nitrite assay and quantification of nitrate release

Nitrite, one stable end product of NO in aqueous solution, was determined by using the Griess method. Of the incubation medium, 50 μl was combined with 50 μl of the Griess reagent (0.1 g of sulphanilamide dissolved in 10 ml of prewarmed 2.5% H_3PO_4 mixed with 0.03 g of naphthylethylenediamine; Sigma), in 10 ml of 2.5% H_3PO_4 in microtitre plates. After 10 min at room temperature, reaction products were determined by spectrophotometry at 540 nm. For calibration of results, a standard curve for $NaNO_2$ was established.

Nitrate release was determined by the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.) based on the conversion of NO_3^- to NO_2^- by nitrate reductase. Subsequent quantification was done using the Griess method described above.

Measurement of L-citrulline

Samples were incubated for 30 min with 45 units/ml urease type VII (Sigma) at 37 °C and proteins were removed by trichloroacetic acid (5%) treatment and subsequent centrifugation. Thiosemicarbazide (5 mg; Sigma) was dissolved in 50 ml of 0.5% diacetylmonoxime (Sigma) and completed with 100 ml of $FeCl_3$ acid (25% H_2SO_4/17% H_3PO_4/0.25% $FeCl_3$). This solution (3 ml) was added to 100 μl of supernatant. After boiling for 5 min at 100 °C, the probes were allowed to cool down at room temperature. The L-citrulline contents were determined by absorbance measurements at 540 nm and data were calibrated by a standard curve.

mRNA analysis

Total RNA was isolated by Trizol Reagent (Gibco-BRL) from RIN cells cultivated in 10 ml culture dishes. Determination and quantification of specific mRNA were performed by reverse transcriptase-PCR, as described elsewhere [18]. Specific primers were used for β-actin (Clontech Laboratories Inc., Palo Alto, CA, U.S.A.) and iNOS [19]. PCR products (β-actin, 27 cycles; iNOS, 35 cycles) were labelled by hybridization with a ^{32}P probe binding at sites between the primer sequences [20]. The signals were quantified by measuring the ^{32}P-stimulated luminescence (PSL) by PhosphorImager. Relative PSL of iNOS was calculated by normalization to the strength of the β-actin signal, set as one.

Western blotting

Cells were washed twice with ice-cold PBS. After lysing in Laemmli sample buffer, the cells were boiled at 95 °C for 5 min and immediately cooled on ice. Subsequent to electrophoresis in SDS/10% PAGE, the gels were blotted on to a nitrocellulose filter (Amersham, Braunschweig, Germany). Filters were blocked for 30 min with Tris-buffered saline (pH 7.4) containing 5% dry milk (Nestlé Deutschland AG, Germany) and incubated for 1 h with a 1:500 dilution of rabbit polyclonal antibody (rabbit anti-iNOS, Dianova, Hamburg, Germany) directed against the inducible form of NOS in rat. The filters were washed with Tris-buffered saline containing 0.06% Tween 20 (Sigma) and incubated for 30 min with peroxidase-labelled mouse anti-rabbit antibody diluted 1:10 000 (Amersham). The detection step was performed with the enhanced chemiluminescence (ECL) detection kit (Amersham). Air-dried filters were exposed to Hyperfilm-ECL (Amersham) and subsequent quantification was done with the Lumi Imager™ (Boehringer Mannheim).

Statistical analysis

Mean values were calculated and are presented with their S. D. values. Data were compared using Student's unpaired or paired t test; $P < 0.05$ indicated a statistically significant difference.

RESULTS

Effect of heavy-metal salts on nitrite production

RIN cells were incubated with increasing concentrations of IL-1, $PbCl_2$ or $HgCl_2$ for 24 h and the supernatant was analysed for nitrite content. As shown in Figure 1, top panel, IL-1 dose-dependently induced nitrite formation whereas exposure to either of the two heavy-metal salts had no effect. Next we tested for synergistic action between IL-1 and $PbCl_2$ or $HgCl_2$. The induction of nitrite production was slightly increased with 0.01 μM $PbCl_2$ and strongly increased in the presence of 10 μM $PbCl_2$ ($P < 0.05$ and $P < 0.001$, respectively). In contrast, there was significant suppression by 0.1–10 μM $HgCl_2$ ($P < 0.05$, Figure 1, bottom panel). In three experiments, nitrate levels were analysed additionally following the reduction to nitrite by nitrate reductase. After stimulation with IL-1β alone, nitrate levels contributed $40.47 \pm 4.49\%$ to total NO_n (nitrate and nitrite), $33.22 \pm 4.66\%$ with 10 μM $PbCl_2$ added and in the presence of 1 μM $HgCl_2$ a proportion of $37.2 \pm 6.1\%$ NO_3^- was found. A significant effect of $PbCl_2$ or $HgCl_2$ on the nitrite/nitrate ratio thus was not observed. At the end of experiments, cell viability was analysed by Trypan Blue exclusion assay. Viability was close to 80% in all cases except for cultures with 10 μM $HgCl_2$, where > 50% of cells were found dead. Therefore, further experiments were conducted with 10 μM $PbCl_2$ and 1 μM $HgCl_2$.

Citrulline was determined as the other product of NO synthase activity. IL-1 treatment of RIN cells dose-dependently increased the citrulline levels in culture supernatants. Addition of $PbCl_2$ significantly enhanced citrulline formation ($P < 0.05$, Table 1), whereas citrulline levels were not altered in the presence of $HgCl_2$.

Table 1 Modulation of L-citrulline-synthesis of RIN cells

Cells (0.5×10^6/ml) were sham-treated or stimulated with 4 or 8 units/ml IL-1β for 24 h; 10 μM $PbCl_2$ or 1 μM $HgCl_2$ was added 3 h prior to cytokine stimulation. Shown are means $\pm$ S.D. of four experiments; *, $P < 0.05$ compared with the respective IL-1β-stimulated cells.

	Citrulline release (μM)		
Treatment	Control	10 μM $PbCl_2$	1 μM $HgCl_2$
PBS	24.1 ± 0.9	29.28 ± 2.15	28.2 ± 1.8
4 units/ml IL-1β	26.58 ± 0.81	38.51 ± 3.76	29.73 ± 1.17
8 units/ml IL-1β	34.63 ± 0.89	51.6 ± 4.7	33.45 ± 2.38

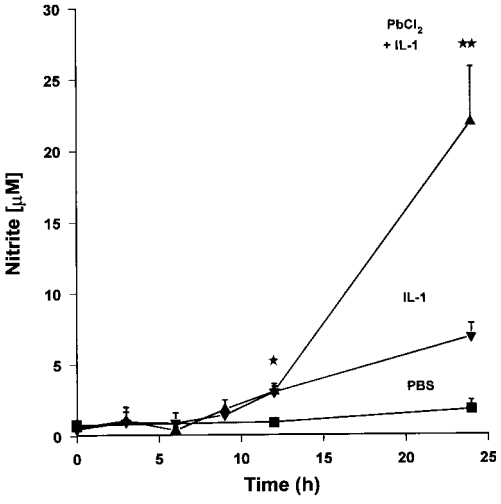

Figure 2 Time course of IL-1β-stimulated nitrite release

Cells were seeded at 0.5×10^6/ml and incubated with 4 units/ml IL-1β (▼), IL-1β + $PbCl_2$ (10 μM) 3 h prior to cytokine stimulation (▲), or were sham-treated (CTL, ■). Supernatants were collected 0, 3, 6, 9, 12 and 24 h after IL-1β stimulation and nitrite was determined. Shown are means $\pm$ S.D. of three experiments; *, $P < 0.05$; **, $P < 0.01$, compared with time point 0 h.

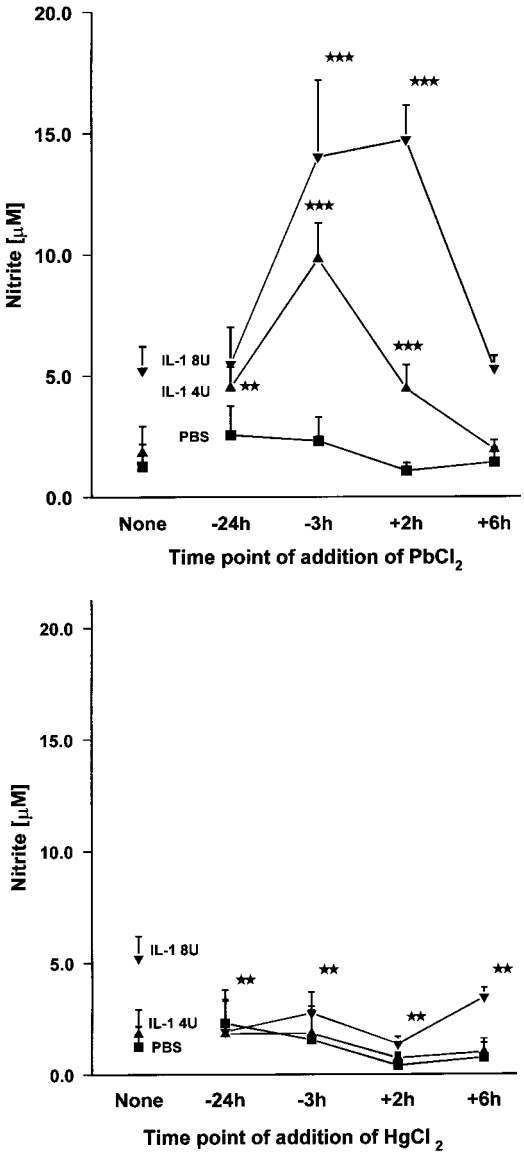

Figure 3 Kinetics of heavy-metal salt effects

(Top panel) Kinetics of Pb^{2+} effect. (Bottom panel) Kinetics of Hg^{2+} effect. Cells (0.5×10^6) were seeded and supernatants were collected 24 h after IL-1β addition for determination of nitrite release. The addition of $PbCl_2$ (10 μM) and $HgCl_2$ (1 μM) was varied from −24 h to +6 h with regard to the time point of cytokine addition. Cells were sham-treated (PBS, ■), stimulated with 4 units (U)/ml IL-1β (▲) or 8 units/ml IL-1β (▼). Control cells were not treated with heavy metals. Shown are means $\pm$ S.D. of at least four experiments; **, $P < 0.01$; ***, $P < 0.001$, compared with cells treated with IL-1β alone.

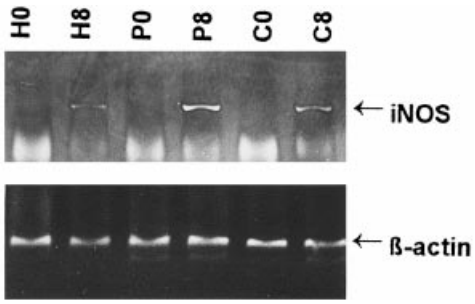

Figure 4 iNOS mRNA levels 6 h after incubation with PBS or 8 units/ml IL-1β + 10 μM $PbCl_2$ or 1 μM $HgCl_2$

Shown are representative data of the experiments described in Table 2. C, PBS-treated cells; P, $PbCl_2$-treated cells; H, $HgCl_2$-treated cells; 8 and 0 represent treatment with 8 units/ml IL-1β or sham-treatment, respectively.

Kinetics of the effect of $PbCl_2$

The enhancing effect of $PbCl_2$ did not affect the kinetics of nitrite formation, i.e. the first significant increase of nitrite in the supernatant was observed at 12 h for $PbCl_2$ + IL-1 as well as for IL-1 alone ($P < 0.05$, Figure 2). The enhancing effect of $PbCl_2$ became evident by 24 h ($P < 0.01$ compared with IL-1 alone, Figure 2).

In another series of experiments, $PbCl_2$ was added to RIN cells at different time points. As shown in Figure 3, top panel, there was a narrow time window during which $PbCl_2$ exerted its potentiating effect on inducible nitrite production, with peak activity from -3 h to $+2$ h in relation to IL-1 treatment. The same kinetic analysis was also performed for the suppressive effect of $HgCl_2$ on inducible nitrite formation. Here the time window was broader, in that $HgCl_2$ was already suppressive when given 24 h before IL-1 (Figure 3, bottom panel). However, as observed before, the modulatory effect of the heavy-metal salt nearly disappeared when added 6 h after the cytokine.

Effect of heavy-metal salts on iNOS gene transcription and translation

RNA was analysed from RIN cells after 6 h of cultivation in the presence of IL-1. By reverse transcriptase-PCR an induction of iNOS mRNA was observed in response to IL-1 (Figure 4). Addition of $PbCl_2$ enhanced the amount of message, whereas exposure to $HgCl_2$ decreased it (Figure 4). PCR products were hybridized to radiolabelled specific probes and quantified on a PhosphorImager. The quantitative data are given in Table 2. After 6 h there was a significant increase of iNOS mRNA when adding $PbCl_2$ to IL-1-stimulated cell cultures ($P < 0.05$), whereas $HgCl_2$ was slightly suppressive (not significant, Table 2). A similar study was performed after 24 h of stimulation with IL-1 (Table 2). At this point of time lower mRNA levels than at 6 h were observed, but the enhancing effect of $PbCl_2$ still persisted ($P < 0.05$).

Finally, we determined whether $PbCl_2$ and $HgCl_2$ altered the amount of iNOS protein synthesized in response to IL-1 treatment. RIN cells were harvested 24 h after stimulation and the extract was analysed by Western blot for the presence of iNOS. IL-1 treatment caused the appearence of a band of 130 kDa, which was reactive with antibodies to iNOS (Figure 5). The amount of iNOS detectable in cells treated with IL-1 + $PbCl_2$ was 7.2-fold more than that detectable in control cells, whereas

Table 2 iNOS mRNA levels at 6 or 24 h after addition of PBS or 8 units/ml IL-1β

Cells were preincubated with $PbCl_2$ (10 μM) or $HgCl_2$ (1 μM) for 3 h prior to IL-1β or PBS addition. Shown are means ± S.D. of at least six experiments. *, $P < 0.05$; **, $P < 0.01$ versus respective IL-1β-treated cells.

	Relative iNOS mRNA (by ^{32}P-stimulated luminescence)		
Treatment	Control	10 μM $PbCl_2$	1 μM $HgCl_2$
After 6 h			
PBS	0.08 ± 0.07	0.04 ± 0.01	28.2 ± 1.8
8 units/ml IL-1β	0.57 ± 0.31	2.83 ± 0.53*	0.25 ± 0.13
After 24 h			
PBS	0.06 ± 0.01	0.04 ± 0.02	0.04 ± 0.03
8 units/ml IL-1β	0.36 ± 0.09	1.08 ± 0.17**	0.15 ± 0.12

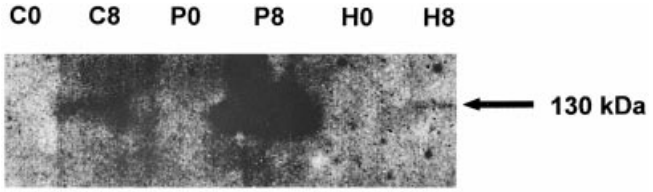

Figure 5 Western-blot analysis of iNOS protein expression

Expression of iNOS of 0.5×10^6 cells/ml was analysed. Shown are the results of control cells ± treatment with 8 units/ml IL-1β (C0, C8) and of cells co-stimulated either with 10 μM $PbCl_2$ (P0, P8) or 1 μM $HgCl_2$ (H0, H8).

exposure to IL-1 + $HgCl_2$ decreased the amounts of iNOS protein by 54.5%, as determined by densitometric evaluation of the Western blot.

DISCUSSION

The data presented demonstrate significant modulatory effects of Pb^{2+} or Hg^{2+} cations on the expression of the iNOS in RIN cells. This β-cell line of rat origin expresses iNOS (NOS II) in response to incubation with IL-1β [21,22]. In contrast with the constitutively expressed isoforms NOS I (neuronal NOS) or NOS III (endothelial NOS), the cytokine-inducible NOS requires synthesis *de novo* and is able to generate NO to a greater extent for a prolonged time period [23–25]. This is in accord with our observation that nitrite levels as a measure of NO production increase not before 6 h after stimulation with IL-1, with a steady further rise until 24 h. There was a clear dose dependency between nitrite levels obtained and the concentration of IL-1β present throughout the dose range analysed; i.e. between 2 and 100 units/ml. This demonstrates that the regulation of iNOS expression in the β-cell line is finely tuned rather than following a simpler either/or mechanism.

We therefore developed the concept that environmental factors might up- or down-regulate the expression of iNOS in β cells. Interestingly, both heavy-metal salts tested significantly modulated IL-1β-induced iNOS expression. Pb^{2+} synergized with suboptimal IL-1 doses and amplified nitrite formation more than three-fold. In contrast, nitrite levels were depressed in the presence of Hg^{2+}. The potentiating effect of $PbCl_2$ was concentration-dependent and discernible from 0.1 μM upward. Sup-

pressive effects of $HgCl_2$ were observed in the same dose range. A clear dose dependency could not be observed, probably because of mercury toxicity, which was substantial at the highest dose (10 μM). One simple explanation of the effects observed would be that Pb^{2+} or Hg^{2+} cations interfere with the oxidation steps during which the NO radical gives rise to the stable products, nitrite and nitrate. We therefore analysed nitrate levels in addition to nitrite. The ratio of IL-1β-induced NO_2^- versus NO_3^- levels was not significantly affected by the presence of $PbCl_2$ or $HgCl_2$. Nitrate levels contributed 33–40 % to total NO_n (nitrite and nitrate), which is in accord with the range of 25–50 % as reported previously for iNOS activity by Stuehr and Marletta [26].

As a further approach we determined citrulline production, the other product of NOS reactivity, in parallel with L-arginine disappearence. Citrulline is not a stable end product of L-arginine oxidation by NOS, but can be reutilized within the cell [27]. Still, the data show a clearly potentiating effect of Pb^{2+} on citrulline formation whereas the suppressive effect of Hg^{2+} was not recognizable.

To determine the time window during which the two heavy metals were able to affect IL-1β-induced NO production, we varied the time points of metal-salt addition with regard to cytokine stimulation. As a result, nitrite formation was affected most when the heavy metals were added between 3 h prior to and 2 h after IL-1, whereas effects were lost when the addition was 6 h after IL-1. Since this narrow time window is overlapping with the period of iNOS gene transcription and translation, the data render a direct interaction between Pb^{2+} or Hg^{2+} and the iNOS protein improbable. These results also confirm our observation that the modulatory effects of Pb^{2+} and Hg^{2+} cations do not involve catalysis of the oxidation reaction of NO to NO_2^-/NO_3^-. Most of the nitrite formation occured between 6 and 24 h, but metal salts were without effect on nitrite levels when present only during this time period. A previous study showed a suppressive effect of both Hg^{2+} and Pb^{2+} cations on NOS I activity in cytosolic extracts of rat brain [28]. However, concentrations required for 50 % inhibition were between 23 μM for $HgCl_2$ and 360 μM for $PbCl_2$. These concentrations were well above those tolerated by cells.

The narrow time window of Pb^{2+} and Hg^{2+} effects suggested that the heavy metals might interfere with transcriptional processes. This hypothesis was tested by analysis of iNOS mRNA formation. Peak levels of iNOS mRNA in response to IL-1β were seen at 6 h, followed by a 50 % decrease at 24 h. Down-regulation of iNOS mRNA is probably due to a negative feedback by NO on iNOS transcription, since NO donors are able to inhibit cytokine-induced iNOS expression in insulin-producing cells [21], glial cells [29] and RAW 264.7 cells [30]. This effect results from the induction or stabilization of iκB, thus preventing the activation of the most important transcription factor for iNOS expression, nuclear factor κB (NF-κB) [31,32]. Quantification of PCR products by PhosphorImaging showed that the addition of $PbCl_2$ led to clearly enhanced levels of iNOS mRNA ($P < 0.05$), whereas $HgCl_2$ decreased the PCR products obtained. The increased amount of iNOS message was paralleled by an increase of iNOS protein in RIN cells exposed to $PbCl_2$. These data identify altered gene expression as the mechanism underlying the modulatory action of Pb^{2+} and Hg^{2+} cations on iNOS activity.

Enhanced iNOS gene expression may be due to an increased transcription rate, due to enhanced mRNA stability, or both. Both mechanisms are established in macrophages, where IFNγ and LPS have been found to enhance iNOS mRNA formation and/or half life [25]. In RIN cells it was reported that IL-1β upregulates iNOS expression by mRNA induction without affecting mRNA stability [24]. It therefore is probable that Pb^{2+} and Hg^{2+} exert their effects via an increased transcription rate. A further argument against modulation of mRNA half life or translation efficiency by Pb^{2+} or Hg^{2+} comes from the observation that the heavy-metal salts are without effect if added after the induction of transcription, i.e. 6 h after IL-1β treatment.

Another important aspect is the apparently tissue-selective action of $PbCl_2$. Blazka et al. [33] reported that Pb^{2+} did not affect iNOS activity in cultured murine brain endothelial cells. In freshly isolated splenic macrophages, a suppressive effect of $PbCl_2$, at concentrations as used here, on iNOS activity was reported [15]. The latter finding suggests opposite effects of Pb^{2+} in macrophages versus β cells. A key difference between murine islet β cells and macrophages with regard to iNOS regulation is at the level of signalling pathways. In murine macrophages, iNOS is readily induced by LPS or synergistically by LPS + IFNγ, but not by IL-1β. In contrast, islet β cells or RIN cells do not respond to LPS or LPS + IFNγ with NO formation, whereas IL-1β is a potent iNOS inducer [34]. The different signalling pathways may provide an explanation for the opposing effects of $PbCl_2$. IL-1-dependent signalling involves more than one pathway in RIN cells. For one, inhibitors of NF-κB such as the protease inhibitor *N*-acetyl-*p*-tosyl-L-lysine chloromethylketone (TLCK) prevent IL-1-induced iNOS expression at the levels of mRNA, protein and NO generation. Secondly, the use of inhibitors of tyrosine kinase like genistein and herbamycin A has indicated that tyrosine phosphorylation events may mediate the IL-1β signal [35–37]. Finally, mitogen-activated protein kinases as part of stress signalling pathways have been shown to contribute to the induction of iNOS by IL-1β in RIN cells [38].

Pb^{2+} has been reported to interact with a number of Ca^{2+}-dependent effector mechanisms based on its ability to substitute Ca^{2+}. Consequently, Pb^{2+} alters the activity of various Ca^{2+}-driven proteins, such as protein kinases C (PKC) and A, adenylate cyclase, phosphodiesterase or calmodulin-dependent protein kinase [39–42]. Especially in the case of PKC, Pb^{2+} is a well-known modulator, capable of both activating and inhibiting the enzyme [43,44]. Although PKC was reported to induce NF-κB in some cells [45,46], it seems not to be a universal activator because other NF-κB-activating agents, such as tumour necrosis factor and IL-1, apparently do not require PKC [47,48]. In RINm5F cells there was only a weak and non-significant effect of the synthetic PKC activator PMA on NF-κB translocation [49], and no effect on iNOS mRNA expression was proved [50]. Additionally, Pb^{2+} was reported to stimulate the phospholipid metabolism in the presence of an ionophore, but not by itself [51]. Finally, Pb^{2+} may also influence protein interactions, including ligand competition and binding affinities, protein folding and the nature of metal-binding sites, as well as rates of protein synthesis and degradation of nuclear acid-binding proteins [52]. In this context, it is of interest that binding of NF-κB to nuclear-responsive elements was decreased progressively by increasing selenite concentrations in cultured T-cells. Selenite inhibition was reversed by addition of dithiothreitol, indicating that this metal as well as Hg^{2+}, Cd^{2+} and Pb^{2+} [53] may react with essential thiol groups required for the actual binding of NF-κB to the promotor region of a target gene. Taken together, the available data suggest that Pb^{2+} and Hg^{2+} target IL-1 signalling rather than iNOS translation or iNOS enzyme activity.

Our finding that $PbCl_2$ increases iNOS expression in an islet β-cell line may be of pathological significance. The current blood level *in vivo* of Pb^{2+} considered to be 'elevated' by the Center of Disease control is 10 μg/dl (0.48 μM) [15], a concentration of Pb^{2+} which was shown here to potentiate the effect of IL-1β on nitrite formation. It therefore is conceivable that chronic exposure

to environmental lead has a significant impact on islet physiology and pathology. The induction of iNOS in β cells *in vivo* has been observed in response to systemically elevated IL-1β levels [54] as well as during islet inflammation preceding the onset of type-I diabetes [55]. In the latter case, intra-islet macrophages may be a major source of IL-1β [34]. Islet endothelial cells are another source of IL-1β [56] and low doses of IL-1β have been shown to potentiate, and high doses to suppress, insulin production via iNOS induction [57–60]. Hence, NO from iNOS may also contribute to the control of endocrine β-cell function. As a consequence, exposure to environmental lead or mercury may modulate β-cell function in normal islets as well as destructive mechanisms in inflamed islets, through the modulation of iNOS expression.

We thank V. Kolb-Bachofen and C. Goebel for reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 503), the Bundesminister für Gesundheit, the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen and the Herbert-Quandt-Stiftung der Varta AG, Hannover.

REFERENCES

1 Stuehr, D. and Marletta, M. A. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7738–7742
2 Stacey, K. J., Sweet, M. J. and Hume, D. A. (1996) J. Immunol. **157**, 2116–2122
3 Welsh, N., Eizirik, D. L., Bendtzen, K. and Sandler, S. (1991) Endocrinology **129**, 3167–3173
4 Corbett, J. A., Wang, J. L., Hughes, J. H., Wolf, B. A., Sweetland, M. A., Lancaster, Jr., J. R. and McDaniel, M. L. (1992) Biochem. J. **287**, 229–235
5 Albina, J. E., Abate, J. A. and Henry, Jr., W. L. (1991) J. Immunol. **147**, 144–148
6 Bogdan, C., Vodovotz, Y. and Nathan, C. (1991) J. Exp. Med. **174**, 1549–1555
7 Vodovotz, Y., Bogdan, C., Paik, J., Xie, Q. W. and Nathan, C. (1993) J. Exp. Med. **178**, 605–613
8 Liew, F. Y. (1995) Curr. Opin. Immunol. **7**, 396–399
9 Rothe, H., Hartmann, B., Geerlings, P. and Kolb, H. (1996) Biochem. Biophys. Res. Commun. **224**, 159–163
10 Habib, A., Bernard, C., Lebret, M., Créminon, C., Esposito, B., Tedgui, A. and Maclouf, J. (1997) J. Immunol. **158**, 3845–3851
11 Mandrup-Poulsen, T., Helqvist, S., Mølvig, J., Wogensen, L. D. and Nerup, J. (1990) Autoimmunity **4**, 191–218
12 Kolb, H. and Kolb-Bachofen, V. (1992) Immunol. Today **13**, 157–160
13 Corbett, J. A. and McDaniel, M. L. (1992) Diabetes **41**, 897–903
14 Haglund, B., Ryckenberg, K., Selinus, O. and Dahlquist, G. (1996) Diabetes Care **19**, 873–875
15 Tian, L. and Lawrence, D. A. (1995) Toxicol. Appl. Pharmacol. **132**, 156–163
16 Tian, L. and Lawrence, D. A. (1996) Toxicol. Appl. Pharmacol. **141**, 540–547
17 Gazdar, A. F., Chick, W. L., Oie, H. K., Sims, H. L., King, D. L., Weir, G. C. and Lauris, V. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 3519–3523
18 Rothe, H., Burkart, V., Faust, A. and Kolb, H. (1996) Diabetologia **39**, 119–122
19 Kleemann, R., Rothe, H., Kolb-Bachofen, V., Xie, Q. W., Nathan, C., Martin, S. and Kolb, H. (1993) FEBS Lett. **328**, 9–12
20 Rothe, H., Faust, A., Schade, U., Kleemann, R., Bosse, G., Hibino, T., Martin, S. and Kolb, H. (1994) Diabetologia **37**, 1154–1158
21 Eizirik, D. L., Cagliero, E., Björklund, A. and Welsh, N. (1992) FEBS Lett. **308**, 249–252
22 Niemann, A., Björklund, A. and Eizirik, D. L. (1994) Mol. Cell. Endocrinol. **106**, 151–155
23 Moncada, S., Palmer, R. M. J. and Higgs, A. (1991) Pharmacol. Rev. **43**, 109–142
24 Eizirik, D. L., Flodström, M., Karlsen, A. E. and Welsh, N. (1996) Diabetologia **39**, 875–890
25 Nathan, C. and Xie, Q. W. (1994) J. Biol. Chem. **269**, 13725–13728
26 Stuehr, D. J. and Marletta, M. A. (1987) Cancer Res. **47**, 5590–5594
27 Hecker, M., Mitchell, J. A., Harris, H. J., Änggård, E. E. and Vane, J. R. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 8612–8616
28 Mittal, C. K. and Houston, D. E. (1995) Toxicologist **15**, 10
29 Park, S. K. and Lin, H. L. (1994) Biochem. Biophys. Res. Commun. **201**, 762–768
30 Nussler, A. K., Billiar, T. R., Liu, Z. Z. and Morris, Jr., S. M. (1994) J. Biol. Chem. **269**, 1257–1261
31 Colasanti, M., Persichini, T., Menegazzi, M., Mariotto, S., Giardano, S., Caldarera, C. M., Sogos, V. and Lauro, G. M. (1995) J. Biol. Chem. **270**, 26731–26733
32 Peng, H. B., Libby, P. and Liao, J. K. (1995) J. Biol. Chem. **270**, 14214–14219
33 Blazka, M. E., Harry, G. J. and Luster, M. I. (1994) Toxicol. Appl. Pharmacol. **126**, 191–194
34 Corbett, J. A. and McDaniel, M. L. (1995) J. Exp. Med. **181**, 559–568
35 Corbett, J. A., Sweetland, M. A., Lancaster, Jr., J. R. and McDaniel, M. L. (1993) FASEB J. **7**, 369–374
36 Welsh, N. (1994) Biosci. Rep. **14**, 43–50
37 Saldeen, J. and Welsh, N. (1994) Biochem. Biophys. Res. Commun. **203**, 149–155
38 Larsen, C. M., Wadt, K. A., Juhl, L. F., Andersen, H. U., Karlsen, A. E., Su, M. S., Seedorf, K., Shapiro, L., Dinarello, C. A. and Mandrup-Poulsen, T. (1998) J. Biol. Chem. **273**, 15294–15300
39 Kern, M. and Audesirk, G. (1995) Toxicol. Appl. Pharmacol. **134**, 111–123
40 Goldstein, G. W. (1993) Neurotoxicology **14**, 97–101
41 Simons, T. J. (1993) Neurotoxicology **14**, 77–85
42 Laterra, J., Bressler, J. P., Indurti, R. R., Belloni-Olivi, L. and Goldstein, G. W. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10748–10752
43 Markovac, J. and Goldstein, G. W. (1988) Nature (London) **334**, 71–73
44 Long, G. J., Rosen, J. F. and Schanne, F. A. (1994) J. Biol. Chem. **269**, 834–837
45 Kleinert, H., Euchenhofer, C., Ihrig-Biedert, I. and Förstermann, U. (1996) J. Biol. Chem. **271**, 6039–6042
46 Feuillard, J., Gouy, H., Bismuth, G., Lee, L. M., Debre, P. and Korner, M. (1991) Cytokine **3**, 257–265
47 Vincenti, M. P., Burrell, T. A. and Taffet, S. M. (1992) J. Cell Physiol. **150**, 204–213
48 Muroi, M. and Suzuki, T. (1993) Cell. Signalling **5**, 289–298
49 Welsh, N. (1996) J. Biol. Chem. **271**, 8307–8312
50 Eizirik, D. L., Cagliero, E., Björklund, A. and Welsh, N. (1992) FEBS Lett. **308**, 249–252
51 Bressler, J., Forman, S. and Goldstein, G. W. (1994) Toxicol. Appl. Pharmacol. **126**, 352–360
52 Goering, P. L. (1993) Neurotoxicology **14**, 45–60
53 Lison, D., Raguzzi, F. and Lauwerys, R. (1990) Pharmacol. Toxicol. **67**, 239–242
54 Wogensen, L. D., Nerup, J., Kolb-Bachofen, V., Kröncke, K. D., Almdahl, T. and Mandrup-Poulsen, T. (1992) Diabetologia **35**, 331–339
55 Rabinovitch, A., Suarez-Pinzon, W. L., Sorensen, O., Bleackley, R. C. and Power, R. F. (1995) J. Immunol. **154**, 4874–4882
56 Steiner, L., Kröncke, K. D., Fehsel, K. and Kolb-Bachofen, V. (1997) Diabetologia **40**, 150–155
57 Zawalich, W. S. and Diaz, V. A. (1986) Diabetes **35**, 1119–1123
58 Comens, P. C., Wolf, B. A., Unanue, E. R., Lacy, P. E. and McDaniel, M. L. (1987) Diabetes **36**, 963–970
59 Wogensen, L. D., Mandrup-Poulsen, T., Markholst, H., Mølvig, J., Lernmark, Å., Holst, J. J., Dinarello, C. A. and Nerup, J. (1988) Acta Endocrinol. (Copenhagen) **117**, 302–306
60 Eizirik, D. L. and Sandler, S. (1989) Diabetologia **32**, 769–773

Received 24 June 1998/24 November 1998; accepted 22 December 1998