Evaluation of islet heme oxygenase-CO and nitric oxide synthase-NO pathways during acute endotoxemia

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Henningsson, Ragnar, Per Alm, and Ingmar Lundquist. Evaluation of islet heme oxygenase-CO and nitric oxide synthase-NO pathways during acute endotoxemia. Am J Physiol Cell Physiol 280: C1242–C1254, 2001.—We investigated, by a combined in vivo and in vitro approach, the temporal changes of islet nitric oxide synthase (NOS)-derived nitric oxide (NO) and heme oxygenase (HO)-derived carbon monoxide (CO) production in relation to insulin and glucagon secretion during acute endotoxemia induced by lipopolysaccharide (LPS) in mice. Basal plasma glucagon, islet cAMP and cGMP content after in vitro incubation, the insulin response to glucose in vivo and in vitro, and the insulin and glucagon responses to the adenylate cyclase activator forskolin were greatly increased after LPS. Immunoblot analysis demonstrated an expression of inducible NOS (iNOS), inducible HO (HO-1), and an increased expression of constitutive HO (HO-2) in islet tissue. Immunocytochemistry revealed a marked expression of iNOS in many β-cells, but only in single α-cells after LPS. Moreover, biochemical analysis showed a time-dependent increase in NO and CO in these islets. Addition of a NOS inhibitor to such islets evoked a marked potentiation of glucose-stimulated insulin release. Finally, after incubation in vitro, a marked suppression of NO production by both exogenous CO and glucagon was observed in control islets. This effect occurred independently of a concomitant inhibition of guanylyl cyclase. We suggest that the impairing effect of increased production of islet NO on insulin secretion during acute endotoxemia is antagonized by increased activities of the islet AMP and HO-CO systems, constituting important compensatory mechanisms against the noxious and diabetogenic actions of NO in endocrine pancreas.

Nitric oxide (NO) is formed from L-arginine under the influence of the nitric oxide synthase (NOS) (29). This enzyme appears in two major isoforms: constitutive Ca2+/calmodulin dependent (constitutive NOS; cNOS) and inducible Ca2+/calmodulin dependent (inducible NOS; iNOS) (23). Both isoforms have been shown to occur in the islets of Langerhans (2, 10, 28, 42). Carbon monoxide (CO) (50) is formed from heme under the influence of the heme oxygenase (HO) enzyme (27), which like NOS, also appears in two isoforms, one inducible HO (HO-1) and one constitutive HO (HO-2) (27). Both these isoforms also exist in the islets of Langerhans (2, 19, 20, 51). It should be added, with regard to cNOS, that this enzyme has two isoforms, a neuronal form (ncNOS) and an endothelial form (ecNOS) (23). These isoforms can be differentiated by immunocytochemistry and immunoblotting (2).

Although NO, produced in small amounts by the cNOS enzyme, is regarded as a putative physiological modulator of islet hormone release (22, 24, 33, 35, 39, 42), there seems to be a general agreement that the large amounts of NO produced by iNOS may have an important role in the pathogenesis of insulin-dependent diabetes mellitus (IDDM) via a noxious influence on β-cells (8, 10, 11, 14, 28, 48). The expression of iNOS in islet β-cells is mediated by the cytokine interleukin (IL)-1β, tumor necrosis factor-α, and interferon-γ, produced by lymphocytes and macrophages that are known to infiltrate the islets during the development of IDDM (8, 12, 14, 48). Furthermore, IL-1β can also induce the expression of heat shock protein 70 and HO-1 in β-cells (47, 51). These proteins, in contrast, are thought to be implicated in islet defense against oxidative stress (29, 47).

NO, when produced by iNOS, is thought to contribute to islet dysfunction and destruction, impairing at several vital sites in the β-cell, such as nuclear DNA, Krebs cycle aconitase, mitochondrial electron transfer chain, and membrane ion channels (13, 14, 28, 48, 49). Moreover, NO has also been shown to induce apoptosis in pancreatic β-cell lines by a cGMP-mediated effect (25).

In the present study, a primary aim was to elucidate possible effects on islet NO production in relation to the function of β-cells and α-cells after in vivo injection of the cytokine-producing endotoxin lipopolysaccharide (LPS). It should be recalled that there is reportedly no effect on iNOS expression in islets directly incubated in vitro with LPS itself (9). Different time points after LPS administration were studied both in vivo and in vitro, because several earlier in vitro investigations on cytokine effects in cultured islets have described both acute stimulatory and later inhibitory effects of differ-

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ent cytokines on insulin secretion (cf. 16, 47, 52). The in vivo route of administration of endotoxin was chosen as a model of islet cell reaction during the “acute phase response,” but also in an attempt to mimic a putative, acute in vivo cytokine attack on the β-cells, an event assumed to be one of several important pathogenic mechanisms in the development of IDDM (16). It should, however, be recalled that the prediabetic state, in many cases, involves a cell-to-cell cytokine delivery over prolonged periods of time and thus might not always be strictly comparable with the present experimental situation. Since we have very recently (19, 20) discovered that the pancreatic islets contain an HO-2 that can produce large amounts of CO, which in turn might be able to inhibit islet NO production (19), we found it of interest to elucidate whether LPS injection would have any influence on islet CO production. Therefore, biochemical analyses were performed to reveal time-dependent changes in the production of NO and CO in islets from LPS-injected animals in relation to islet hormone secretory capacity both in vitro and in vivo. In certain experiments, we also measured perturbations in islet cAMP and cGMP levels as well as changes in protein expression of the different isoforms of the NOS and HO proteins with the use of immunoblotting and immunocytochemistry.

MATERIALS AND METHODS

**Animals.** Female mice of the NMRI strain (B&K, Sollentuna, Sweden) weighing 28–37 g were used in all studies. They were fed a standard pellet diet (B&K) and tap water ad libitum. All animals used for preparation of pancreatic islets were killed by a blow to the neck, whereupon the pancreatic glands were resected with the tail. Sections were incubated overnight with rabbit antisera to HO-2 (code OSA 200, lot 709422; 1:500; both antisera were purchased from StressGen Biotechnol, Victoria, BC, Canada), or ncNOS (1:2,400) (2, 3). The selectivity of the presently employed HO-1 and HO-2 antisera as well as appropriate control experiments were previously reported (2, 20). After being rinsed, the sections were incubated in Texas red-conjugated donkey anti-rabbit immunoglobulins (IgG) for 90 min, rinsed, and mounted.

For the simultaneous demonstration of two antigens, sections were incubated overnight with antisera raised in guinea pigs to insulin (1:1,600) or glucagon (1:4,000; both antisera were purchased from Linco, St Louis, MO). After being rinsed, the sections were incubated for 90 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG and rinsed. Immediately after that, some of the sections were incubated with Texas red-conjugated IgG (1:125), rinsed, and mounted (see above). For the demonstration of iNOS, other sections were mounted, and the insulin and glucagon immunoreactivities were documented by microscopy. After being rinsed overnight in phosphate-buffered saline (PBS) with elimination of the covergrips, the sections were incubated for 10 min in 5% swine serum in PBS and then overnight in rabbit antisera to iNOS (1:2,000) (17, 26). After being rinsed, the sections were incubated with biotinylated swine anti-rabbit IgG (1:200 for 30 min) and then with peroxidase-coupled avidin (1:1,000 for 30 min). After being rinsed, the immunoreactive products were detected by incubation for 5 min in a solution containing 25 μg of 3,3'-diaminobenzidine, 100 ml of PBS, and 50 μl of hydrogen peroxide. After being rinsed in running tap water, the sections were dehydrated and mounted.

An Olympus × 50 fluorescence microscope, equipped with epi-illumination and appropriate filter settings for Texas red and FITC immunofluorescence, was used for the examinations of the sections (31).

The primary and secondary antisera were diluted in PBS. In control experiments, no immunoreactivity could be detected in sections incubated in the absence of the primary antisera or with antisera absorbed with excess of the corresponding immunizing antigen (100 μg/ml). No absorption controls could be performed with the iNOS antisera because antigenic substances were not available. The characteristics of the iNOS and the ncNOS antisera have been presented previously (3, 26, 45).

In vivo experiments. LPS and d-glucose were dissolved in 0.9% NaCl (saline). Forskolin was dissolved in DMSO and then diluted in saline. LPS was dissolved in saline and then injected intraperitoneally (10 mg/kg), whereas glucose (3.3 mmol/kg) and forskolin (7.3 μmol/kg) were injected intravenously in a tail vein (volume load: 5–10 μl/g mouse). Controls received either saline (glucose) or vehicle (forskolin). All animals were fed freely during the whole study. Blood sampling was performed as described previously (37). The concentrations of insulin and glucagon in plasma were determined by radioimmunoassay (1, 18, 36). Plasma glucose concentrations were determined enzymatically (7).

**Assay of islet NOS activity.** Isolated, handpicked, and thoroughly washed islets were collected in ice-cold buffer (840 μl) containing 20 mmol/l HEPES, 0.5 mmol/l EDTA, and 1 mmol/l dithiothreitol, pH 7.2, and immediately frozen at −20°C. On the day of assay, the islets were sonicated on ice, and the buffer solution containing the islet homogenate was supplemented to also contain 0.45 mmol/l CaCl2, 2 mmol/l NADPH, 25 units of calmodulin, and 0.2 mmol/l L-arginine in a total volume of 1 ml as previously described (39). The buffer composition is essentially the same as previously described for assay of NOS in brain tissue using radiolabeled L-arginine (6). The homogenate was then incubated at 37°C under
constant air bubbling, 1.0 ml/min, for 3 h. It was ascertained that under these conditions, the reaction velocity was linear for at least 6 h. Aliquots of the incubated homogenate (200 μl) were then passed through a 1-ml Amprep CBA cation-exchange column for high-performance liquid chromatography analysis. The amount of l-citrulline formed was then measured in a Hitachi F-1000 fluorescence spectrophotometer (Merck) as previously described (39). NO and l-citrulline are produced in equimolar concentrations. The nitrogen atom of the guanidino group of l-arginine is released as NO, which is highly reactive, and therefore the simultaneous liberated and stable l-citrulline is preferably measured (6, 29). The methodology has been described in detail earlier (21, 39). Protein was determined according to Bradford (5) on samples from the original homogenate.

Western blot analysis. Approximately 200 islets were handpicked in Hanks’ buffer under a stereomicroscope and sonicated on ice (3 × 10 s). The protein content was determined according to Bradford (5). Homogenate samples representing 20 μg of total protein from islet tissue were run on 10% SDPAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by electro-transfer (10- to 15 V, 60 min; semi-dry transfer cell; Bio-Rad, Richmond, CA). The membranes were blocked in 9 mM Tris·HCl (pH 7.4) containing 5% nonfat milk powder for 40 min at 37°C. Immunoblotting with rabbit anti-mouse nNOS (N-7155), iNOS (N-7782; 1:2,000; Sigma), HO-2, and HO-1 (1:2,000) antibodies (StressGen Biotechnol) was performed for 16 h at room temperature. The membranes were washed twice and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000; Sigma) for 90 min. Antibody binding to nNOS, iNOS, HO-2, and HO-1 was detected using 0.25 mM CDP-Star (Boehringer Mannheim, Mannheim, Germany) and the signal enhancer Nitro Block II (Tropix, Bedford, MA) for 5 min at room temperature. The chemiluminescence signal was visualized by exposing the membranes to DuPont Cronex X-ray films for 1- to 5 min.

Hormone secretion in vitro. Freshly isolated islets were preincubated for 30 min at 37°C in Krebs-Ringer bicarbonate buffer, pH 7.4, supplemented with 10 mmol/l HEPES, 0.1% bovine serum albumin, and 1 mmol/l glucose. Each incubation vial contained 10 islets in 1.0 ml buffer solution, and, unless otherwise stated, was gassed with 95% O2-5% CO2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed to a medium containing either 1.0, 7.0, or 16.7 mmol/l of glucose together with the different test agents, and the islets were incubated for 60 min. Aliquots of the medium were then removed and frozen for subsequent assays of insulin (18) and glucagon (1, 36).

Measurement of islet HO activity. CO production was determined according to Bradford (5). Homogenate samples representing 20 μg of total protein from islet tissue were run on 10% SDPAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membranes by electro-transfer (10- to 15 V, 60 min; semi-dry transfer cell; Bio-Rad, Richmond, CA). The membranes were blocked in 9 mM Tris·HCl (pH 7.4) containing 5% nonfat milk powder for 40 min at 37°C. Immunoblotting with rabbit anti-mouse nNOS (N-7155), iNOS (N-7782; 1:2,000; Sigma), HO-2, and HO-1 (1:2,000) antibodies (StressGen Biotechnol) was performed for 16 h at room temperature. The membranes were washed twice and then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:10,000; Sigma) for 90 min. Antibody binding to nNOS, iNOS, HO-2, and HO-1 was detected using 0.25 mM CDP-Star (Boehringer Mannheim, Mannheim, Germany) and the signal enhancer Nitro Block II (Tropix, Bedford, MA) for 5 min at room temperature. The chemiluminescence signal was visualized by exposing the membranes to DuPont Cronex X-ray films for 1- to 5 min.

Effect of LPS on basal plasma levels of insulin, glucagon, and glucose. To investigate putative effects of LPS on plasma levels of insulin, glucagon, and glucose in relation to time, the plasma concentrations of these parameters were determined at different time points (1, 2, 4, 6, and 24 h after intraperitoneal injection of LPS). Figure 1 shows that from 4 h and onward after LPS injection, the plasma glucose levels were suppressed compared with controls. The plasma glucagon levels started to rise already at 2 h after LPS injection and remained elevated throughout. No apparent changes in the plasma insulin levels were observed. To investigate the possibility of a long-lasting, LPS-induced impairment of basal glucose homeostasis, the plasma glucose levels were also determined at 11 days after LPS injection. In LPS-injected animals, the basal plasma glucose concentration was 9.0 ± 0.3 mmol/l (n = 8) vs. 9.7 ± 0.4 mmol/l (n = 8) in saline-injected animals, indicating no apparent impairment of basal glucose homeostasis at this time point following a single injection of LPS.

In vivo effects of glucose and forskolin on insulin and glucagon responses at 3 and 6 h after LPS injection. The acute in vivo effects of saline (basal values), glucose, or forskolin administration on insulin and glucagon responses after LPS treatment vs. controls were investigated at 3 and 6 h after injection of LPS, i.e., at time points when initial changes in the basal plasma levels of glucagon and glucose were observed. Figure 2 (the four bars to the left) shows that the basal plasma glucagon levels were greatly enhanced at both time points (3 and 6 h) in LPS-treated mice, whereas the
insulin levels were unaffected. Furthermore, the basal plasma glucose concentrations were suppressed in the LPS mice. Figure 2 (the four bars in the middle) shows that in the LPS mice, the acute insulin response (at 2 min) after an intravenous challenge of glucose was increased at both 3 and 6 h after LPS treatment, compared with glucose-injected controls. Glucose injection, however, did not acutely suppress the elevated levels of glucagon. Since activation of the cAMP system in the β-cell is known to potentiate glucose-stimulated insulin release, influence of LPS administration on this system was studied by measuring the acute insulin and glucagon response following an intravenous injection of the adenylate cyclase activator forskolin. Figure 2 (the four bars to the right) shows that LPS-treated animals displayed a greatly increased acute insulin and glucagon response to forskolin at 3 h after LPS. This increased response was further exaggerated when forskolin was injected at 6 h after LPS administration.

Islet NOS activity after LPS injection. Figure 3A shows the production of NO in islets isolated at 3, 6, 16,
24, and 48 h after LPS injection. At 6 h after LPS administration, there was a clear increase in NO production that was further increased with a peak value at 16 h, representing a threefold increase compared with controls (75.2 ± 6.2 vs. 22.8 ± 1.3 pmol min⁻¹ mg⁻¹ protein⁻¹). The NO production was still increased at 2 days after LPS administration (Fig. 3A).

**Western blot of NOS isoforms.** In islets from mice treated with LPS (20 h after LPS administration), there was an exclusive expression of iNOS protein (Fig. 3B), whereas none could be detected in islets from controls given saline. In contrast, expression of ncNOS protein could be detected in islets from both control and LPS-treated animals (Fig. 3B).

**Effects of LPS injection on insulin and glucagon secretion from isolated islets in the presence of low and high concentrations of glucose.** Insulin secretion stimulated by a high concentration (16.7 mmol/l) of glucose was greater from islets of LPS-treated animals than from control islets at 3, 6, and 16 h after LPS administration (Fig. 4A). No appreciable differences in insulin secretion between the two groups could be seen in the presence of the low concentration of glucose. In comparison, with regard to glucagon release, no apparent differences were noted at either low (glucagon stimulating) or high glucose concentration after LPS treatment (Fig. 4B).

**Effects of in vitro NOS inhibition on hormone release from islets isolated from LPS- or saline-treated mice.** In the presence of 16.7 mmol/l glucose, the NOS inhibitor L-NAME (1.0 and 5.0 mmol/l) potentiated the secretion from control islets at 3, 6, and 16 h after LPS administration (Fig. 4A). No appreciable differences in insulin secretion between the two groups could be seen in the presence of the low concentration of glucose. In comparison, with regard to glucagon release, no apparent differences were noted at either low (glucagon stimulating) or high glucose concentration after LPS treatment (Fig. 4B).
of insulin from islets of both control and LPS-treated mice (Fig. 5A). At a concentration of 5 mmol/l of L-NAME, the rate of insulin secretion from islets of LPS-treated mice was markedly enhanced; i.e., 18.0 ± 0.87 nmol/islet⋅h⁻¹ compared with 9.71 ± 0.89 nmol/islet⋅h⁻¹ in control islets. Glucagon secretion from control islets, but not from LPS islets, was inhibited by L-NAME (Fig. 5B).

Effect of LPS injection on hormone release from isolated islets stimulated by the adenylate cyclase activator forskolin. The insulin secretory response to forskolin in the presence of a basal concentration of glucose (7 mmol/l) was four- to sevenfold greater from incubated islets isolated from LPS-treated mice compared with islets from control mice (Fig. 6). This difference could be seen at all time points tested (3, 6, 16, and 24 h after LPS treatment), with the peak value at 24 h. The glucagon secretion was also greater in islets from LPS-treated animals than from controls. In contrast to the insulin release, the potentiation of the glucagon release was more pronounced at the early time points (3 and 6 h) and declined with time.

Fig. 5. Effect of the NOS inhibitor N⁵G-nitro-L-arginine methyl ester (L-NAME; 1.0 and 5.0 mmol/l) on the secretion of insulin (A) and glucagon (B) from islets isolated from control or LPS-treated mice (10 mg/kg) at 20 h after injection of LPS or saline. Glucose concentration in the incubation medium was 16.7 mmol/l. Means ± SE are shown on each point for 8 to 10 batches of islets obtained from 2 separate experiments. Incubation time was 60 min. Random difference between the different control groups (open bars) and the LPS-treated groups (filled bars) at 1.0 and 5.0 mmol/l L-NAME concentration: ***P < 0.01. Stars denote difference between control groups (open bars) in absence vs. presence of 1.0 and 5.0 mmol/l L-NAME: ●P < 0.05; ***P < 0.001.

Fig. 6. Secretion of insulin (A) and glucagon (B) in the presence of 7 mmol/l of glucose (G 7.0) or 7.0 mmol/l of glucose + forskolin 20 μmol/l (G 7.0 + F) from islets isolated from either control or LPS-treated mice (10 mg/kg) 3, 6, 16, and 24 h after injection of LPS or saline. Means ± SE are shown on each point for 8 to 12 batches of islets obtained from 8 separate experiments. Incubation time was 60 min. Random difference for controls + forskolin vs. LPS + forskolin: ***P < 0.001; random difference for controls (G 7.0) vs. control + forskolin (G 7.0 + F): *P < 0.05; **P < 0.01.
Immunocytochemical findings. NeNOS immunoreactivity was observed in the majority of islet cells. Double immunolabeling showed that most neNOS immunoreactive cells also expressed insulin immunoreactivity (Fig. 7, A and B). Glucagon immunoreactivity occurred in a smaller number of islet cells, mostly located in the periphery, and some of these also displayed ncNOS immunoreactivity (Fig. 7, C and D). Note the absence of ncNOS immunoreactivity in the surrounding exocrine tissue. After LPS administration, no overt change of ncNOS immunoreactivity in islet cells could be seen (data not shown).

As also previously reported (14), most islet cells displayed HO-2 immunoreactivity, whereas no HO-1 immunoreactivity could be observed (data not shown). After LPS administration, no detectable change in the pattern of HO-1 or HO-2 immunoreactivity in islet cells was found compared with untreated mice (data not shown).

No iNOS immunoreactivity was seen in islets of untreated mice (Fig. 8A). In contrast, after LPS administration, iNOS immunoreactivity could be detected. At 3 and 6 h after LPS injection, weak iNOS immunoreactivity was observed in a small number of cells, compared with at 16 and 24 h after LPS injection, when iNOS immunoreactivity with a strong intensity occurred in a comparatively larger number of cells, which were diffusely scattered over the islets (Fig. 8, B and D). Most of the iNOS immunoreactive cells also expressed insulin immunoreactivity (Fig. 8, B and C), whereas iNOS immunoreactivity could convincingly be detected only in single glucagon immunoreactive cells (Fig. 8, D and E).

In control experiments, iNOS immunoreactivity was observed in LPS-induced tissues (macrophages in lung and liver) in which no ncNOS immunoreactivity could be visualized (data not shown).

Islet HO activity after LPS injection. HO activity measured as production of CO in isolated islets at 3, 6, 20, and 48 h after intraperitoneal LPS administration is shown in Fig. 9A. The production of CO was significantly increased at 6 h (50%) after LPS treatment and then further elevated after 20 (120%) and 48 (130%) h compared with controls.

Western blot of HO isoforms. Expression of HO-2 at 20 h after LPS administration was seen in islets of both saline- and LPS-injected animals. Notably, the expression of HO-2 protein was stronger after LPS than in controls (Fig. 9B). Although the blots are only semiquantitative, the visual increase in HO-2 protein expression was in good accordance with the marked increase of CO production at 20 h (Fig. 9A). Expression of HO-1 protein could be detected only in LPS-treated animals (Fig. 9B).

Influence of LPS injection on islet cAMP and cGMP content. Islets taken from mice 20 h after LPS treatment and then incubated in the presence of 1 mmol/l glucose for 1 h showed a 75% higher content of cAMP than control islets (Table 1). Incubation of islets at a high concentration of glucose (16.7 mmol/l) increased cAMP levels almost sixfold in control islets but only threefold in LPS-treated islets. At this high concentra-
tion of glucose, no differences could be seen in islet cAMP content after LPS treatment compared with controls. cGMP levels were significantly greater (55%) in islets from LPS-treated mice than in islets from control mice after incubation at both a low (1 mmol/l) and a high (16.7 mmol/l) concentration of glucose (Table 1).

**Effect of glucagon and exogenous CO in the absence and presence of the guanylyl cyclase inhibitor ODQ on islet NOS activity and insulin and glucagon secretion.** The most prominent effects recorded following LPS injection, apart from the increase in islet NO evolution, were a marked increase in plasma glucagon levels (Fig. 1B) as well as a great stimulation of islet CO production (Fig. 9A). In view of these findings, the effects of glucagon and CO on NOS activity in normal control islets were tested. The production of NO in islets incubated at 16.7 mmol/l of glucose was significantly decreased both by means of exogenously applied CO (10 μmol/l) and glucagon (10 μmol/l; Fig. 10A). Preparation of CO solutions and estimation of concentration were previously described (32). CO was still able to inhibit NO production in the presence of the guanylyl cyclase inhibitor ODQ (10 μmol/l). Both glucagon and CO were stimulatory to insulin secretion (Fig. 10B), and CO also stimulated glucagon secretion (Fig. 10C). The stimulatory effects of CO on insulin and glucagon secretion were inhibited in the presence of ODQ (Fig. 10, B and C).
Fig. 9. A: heme oxygenase (HO) activity in isolated islets taken from mice at 3, 6, 20, and 48 h after an intraperitoneal LPS (10 mg/kg) injection. Controls were injected with saline at the different time points. Data are expressed as carbon monoxide (CO) formation (pmol · mg protein⁻¹ · min⁻¹). Values are means ± SE for 3 to 4 pools of islets at the different time points. Each pool was taken from 1 to 2 mice in the different groups. HO activity in islets from control animals did not differ between the different time points, and, therefore, all control values are presented under time 0. Random difference vs. control islets: *P < 0.05, ***P < 0.001. B: Western blots in islets at 20 h after injection of LPS or saline (controls). Lanes 1 and 2 incubated with constitutive HO (HO-2) antibody; lanes 3 and 4 incubated with inducible HO (HO-1) antibody. Lanes 1 and 3: 20 μg of islet protein from control mice; lanes 2 and 4: 20 μg of islet protein from LPS-treated mice (10 mg/kg). Molecular weights indicated in the figure.

DISCUSSION

In this study, we have presented a model of islet cell reaction during the acute phase response after LPS injection. A single high dose of LPS had no appreciable effect on basal plasma levels of insulin during the time period studied (1 to 24 h), whereas basal glucagon levels started to increase at 2 h and remained elevated throughout, probably to counterbalance an apparent decrease in plasma glucose levels. The in vivo insulin response to glucose was already markedly increased at 3 h after LPS injection. At this time point, both the insulin and glucagon responses to the adenylyl cyclase activator forskolin were greatly exaggerated both in vivo and in vitro, suggesting that an early event induced by the LPS-cytokine attack on the pancreatic islets is manifested as a marked increase in the sensitivity and capacity of the islet cAMP pathway. This enhanced secretory capacity of the islet cAMP system increased with time in insulin-producing β-cells but decreased in glucagon-producing α-cells. In β-cells, such a temporal pattern may be brought about as a compensatory mechanism against the accompanying gradual increase in the production of islet NO, which is known to greatly impair glucose-stimulated insulin release (9, 14). In contrast, according to ancillary experiments in our laboratory, NO has no inhibitory effect on insulin release stimulated by the phosphodiesterase inhibiting and thus cAMP-stabilizing agent IBMX. Moreover, NO itself is stimulatory to glucagon release (21, 33, 39–41) and thus there is probably no further need for stimulation of the α-cell cAMP system during the latter part of the time period studied. In addition, the production of islet CO, which in contrast to NO is stimulatory to insulin release (19, 20), was temporally increased, almost in parallel to the NO production and thus similar to the cAMP system, possibly serving as a compensatory mechanism against the impairing effect of NO on the β-cells. It is notable that activation of the cAMP system seems to be more rapid in onset than the activation of the HO-CO system. Islet NO production slightly declined after 16 h but was still twofold elevated at 48 h, whereas CO production was maximally elevated at both 20 and 48 h. This pattern could be a reflection of our recent suggestion (19) and our present demonstration (Fig. 10A) that CO has a direct inhibitory action on islet NOS activity. Therefore, “normalization” of glucose-stimulated insulin release in isolated islets at 24 h after LPS injection could represent a balance between the inhibitory effect of NO and the stimulatory effects of CO and cAMP, the cAMP system in β-cells being continuously stimulated by the enhanced intra-islet glucagon concentrations following the increased glucagon release and also in turn by the long-lasting elevation of the plasma glucagon levels. It should be noted that the in vivo glucagon release was much more pronounced than in vitro, suggesting that neural stimulation is involved in the LPS-injected animals. It seems conceivable that these animals are subjected to acute stress during the initial stage of endotoxemia, possibly involving adrenergic stimulation of glucagon release (43). The very high plasma glucagon levels are also,

| Table 1. Content of cAMP and cGMP in islets isolated 20 h after saline or LPS injection and then incubated in presence of low or high concentrations of glucose |
|---|---|---|
| Islet cAMP, fmol/islet | Islet cGMP, amol/islet |
| Controls, 1.0 mmol/l glucose | 7.86 ± 0.96 | 306.7 ± 29.1 |
| LPS, 1.0 mmol/l glucose | 13.68 ± 0.76* | 474.0 ± 13.5† |
| Controls, 16.7 mmol/l glucose | 40.96 ± 2.38 | 588.7 ± 72.0 |
| LPS, 16.7 mmol/l glucose | 42.37 ± 3.80 | 909.6 ± 76.4* |

Values are means ± SE for 5 to 8 pools of islets. Each pool was taken from 3 to 4 animals in each group. Random difference for controls vs. lipopolysaccharide (LPS)-treated islets in the presence of the same concentration of glucose: *P < 0.01, †P < 0.05.
most probably, a defense against the LPS-induced hypoglycemia, which, in turn, largely seems to be the result of an increased peripheral glucose utilization being, at least partly, mediated by an increased NO production in these tissues (38). Hence, during the beginning of this initial LPS period, the increased sensitivity to glucose stimulation in the β-cell most probably depends on an increased cAMP activity and later on the additional activation of the CO production. As discussed below, both glucagon and CO directly suppress islet NO production, resulting in a beneficial effect on glucose-stimulated insulin release. During the later stage of the endotoxemic time period studied, i.e., at 24 to 48 h, it cannot be excluded that a reduced food intake (manifested as a significant weight loss at 48 h) could have influenced our results. However, because fasting is known to greatly reduce basal and glucose-stimulated insulin release, this possibly only has a marginal effect since insulin release was normal or enhanced, even in the face of hypoglycemia.

Immunocytochemistry and immunoblots showed a significant expression of iNOS in islet tissue at 16 h after LPS treatment, whereas no iNOS activity was detected in islets of control mice. iNOS immunoreactivity was most convincingly seen in many insulin-producing β-cells, but only in single glucagon-producing α-cells. This pattern is in perfect accordance with our very recent immunocytochemical findings in the rat endocrine pancreas, where we also used confocal microscopy (2). Sixteen hours after LPS, we found that NO production was increased threefold. The inducible isofrom of NOS is known to produce much larger amounts of NO than cNOS. Thus NO production by iNOS is continuous and elicited during long time periods, whereas cNOS-derived NO is manifested in small NO bursts (44). In the present study, NO production was still doubled at 48 h after in vivo LPS administration and probably derived mostly from iNOS. In this context, it should be recalled, as mentioned earlier in this paper, that direct addition of LPS to isolated islets reportedly has only negligible effects on NO production (9) and thus the increase of islet NOS activity in the present study is most probably a result of LPS-stimulated cytokine production in vivo.

In agreement with earlier studies in the rat (52), the present data indicate that the insulin secretory machinery is somehow sensitized to glucose after in vivo LPS treatment and that endotoxic shock can be accompanied by hypoglycemia (4). Most of the effects on islet hormone secretion were already manifested during the first 6 h after LPS injection. It is notable that glucose-stimulated insulin release was increased not only in the face of an increased islet NO production but also during a gradual decrease of the plasma glucose levels. In fact, islets of LPS-injected mice displayed a normal glucose-stimulated insulin release at 24 h, when the animals suffered weight reduction and a profound hypoglycemia. This underlines the importance of the regulatory and compensatory system(s) that could contribute to restoring secretion of insulin during the increased production of NO, which, as mentioned earlier in this paper, has been shown to be a powerful inhibitor of glucose-stimulated insulin secretion (14, 19, 40, 41).

Fig. 10. A: NOS activity in control islets incubated at 16.7 mmol/l of glucose in absence and presence of glucagon (10 μmol/l) or CO (10 μmol/l), alone or together with the guanylyl cyclase inhibitor 1H-[1,2,4]oxidiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μmol/l). NO production was measured as the equivalent of L-citrulline formation (29) (pmol·min⁻¹·mg protein⁻¹). Groups of 200 islets/1.5 ml of medium were incubated for 60 min. Data are means ± SE for 4 to 5 batches of islets. Random difference vs. control: *P < 0.05; **P < 0.01; ***P < 0.001. B and C: secretion of insulin (B) and glucagon (C) from isolated islets in the same experiment. Random difference vs. control: *P < 0.05; **P < 0.01; random difference as indicated in the figure: *P < 0.05; N.D. = not determined.
Hemin has been shown to protect against IL-1β-induced inhibition of islet function in the rat, probably by scavenging NO and/or increasing the resistance to NO production (49), and, like NO, CO is able to bind to heme-containing enzymes (the different NOS isoforms) and regulate the activity of these enzymes. Islet CO production after LPS injection was time dependently increased, supporting the idea that the activity of islet HO is increased as a consequence of the action of increased levels of cytokines brought about by LPS administration. Interestingly, this increased CO production seemed to be derived from both the acutely expressed HO-1 protein as well as from an enhanced amount of the HO-2 protein (cf. Fig. 9). It should be noted, however, that the immunocytochemical techniques used did not seem to be sensitive enough to detect these changes. A few reports indicate that NO itself, at least in other tissues, is responsible for the induction of HO-1 and that NO can increase CO production via binding to the heme moiety of HO (30). In addition to CO, equimolar concentrations of biliverdin is produced during the HO-mediated heme degradation. Biliverdin is then degraded to bilirubin, also known as a strong antioxidant. All these data indicate that the newly discovered HO-CO pathway within the islets of Langerhans (19, 20) might constitute an important defense mechanism against oxidative stress and against the deleterious effects of NO.

In the search for other possible compensatory mechanisms of keeping insulin secretion almost normal during endotoxemia, we found a remarkable, high secretory response of insulin and glucagon to the adenylate cyclase activator forskolin after LPS treatment. In islets of LPS-treated mice, forskolin-induced insulin secretion was four to seven times higher than in islets of untreated mice, depending on the time interval after LPS administration. This, together with the observation of an increased content of cAMP in islets of LPS-treated animals, suggests that there might be an up-regulation and/or increased sensitivity of the islet cAMP protein kinase A system during the development of such an endothelin-derived type of islet dysfunction. In fact, our present data suggest that this compensatory system is more rapid in onset than is the compensatory mechanisms exerted by the HO-CO system. It should be noted in this context that during in vitro culture, IL-1β and NO donors have earlier been shown to decrease the cAMP content in rat islets (16). In contrast, as shown by our present data in vivo as well as in vitro using freshly isolated islets, there seems to be an increased capacity of the secretory pathway(s) mediated by the islet cAMP system, which together with the HO-CO system could be at least partly responsible for islet compensatory mechanisms against increased NO production.

cGMP levels were also significantly increased after LPS treatment. This could be due to both an increased NO and an increased CO production, since both gases are known as potent guanylyl cyclase activators, by binding to the heme moiety of the enzyme. In this context, it should be noted that CO is reportedly considerably less potent than NO as an activator of guanylyl cyclase (12). Earlier studies described both a large arginine dependent and a small arginine-independent increase in cGMP in rat islets after in vitro exposure to IL-1β (15). The function of a guanylyl cyclase-cGMP protein kinase G-activating system in the islets of Langerhans, as well as the function of NO itself, are not clearly elucidated, although cGMP is reported as a potent mediator of long-term, NO-induced apoptosis in β-cells (25). In contrast, we have recently suggested that cGMP might be at least partly responsible for mediating acute CO-stimulated insulin and glucagon secretion (19). Regarding NO, the stimulating effect on the cGMP system, through its binding to the heme group of guanylyl cyclase in the β-cell, is likely to be strongly counteracted and overshadowed by its ability to induce formation of S-nitrosothiols (46), which apparently negatively modulates stimulus-secretion coupling of nutrient-induced insulin secretion (21, 34, 39–41).

Islet NOS activity was markedly decreased after addition of either glucagon or CO to control islets (cf. Fig. 10). The NOS inhibitory effect exerted by CO does not seem to be mediated by its stimulatory effect on glucagon release, since guanylyl cyclase inhibition by ODQ, which extinguished the stimulation of glucagon secretion induced by CO, did not affect its ability to inhibit NO production. This finding might have a possible clinical application in the future. People developing islet dysfunction during acute or long-term, endotoxemia-induced cytokine production might be helped with glucagon and/or a putative “CO-promoting” therapy to suppress the deleterious effects of the increased NO evolution.

In summary, isolated islets from LPS-treated mice displayed expression of iNOS and HO-1 proteins and an increased expression of HO-2 protein concomitant with increased production of NO and CO. Immunocytochemistry demonstrated that LPS-induced iNOS immunoreactivity could be convincingly seen in many insulin-producing β-cells, but only in single glucagon-producing α-cells. ncNOS immunoreactivity could be readily detected in both α- and β-cells but was seemingly unaffected by LPS. Moreover, glucose-stimulated insulin release was not impaired despite the well-known negative influence of NO on nutrient-induced insulin secretion. This could be explained by both an increased activity and sensitivity of the cAMP system and an increased CO production in islets from LPS-treated animals. The beneficial (inhibiting) effects of glucagon and CO on islet NOS activity may have important implications regarding possible future treatment of endotoxemia-induced dysfunction of the pancreatic islets.

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