

Cytokine- or Chemically Derived Nitric Oxide Alters the Expression of Proteins Detected by Two-Dimensional Gel Electrophoresis in Neonatal Rat Islets of Langerhans

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Interleukin-1 β (IL-1 β) treatment of neonatal rat islets for 24 h induces changes in the expression of 105 of 2,200 proteins, as determined previously by two-dimensional (2D) gel electrophoresis. Nitric oxide (NO) has been implicated as one of the mediators of IL-1 β effects in insulin-containing cell lines and rat islets. The aims of this study were 1) to determine the involvement of NO in IL-1 β -induced alterations in protein expression and 2) to investigate the effects of chemically generated NO on protein expression by 2D gel electrophoresis of neonatal rat islet samples. IL-1 β -induced NO production was prevented by incubation of islets in arginine-free medium supplemented with the arginine analog N_G -nitro-L-arginine. [35 S]methionine-labeled islet proteins were separated using 2D gel electrophoresis and analyzed using the BioImage computer program. Analysis revealed that the expression levels of 23 protein spots of the 105 protein spots, altered by prior treatment with IL-1 β (60 U/ml) alone, were significantly affected ($P < 0.01$ [$n = 4$] and $P < 0.05$ [$n = 19$]) when NO production was prevented. The effects of chemically generated NO were investigated by exposing islets to the NO donor GSNO (100 μ mol/l) for 24 h before labeling with [35 S]methionine and 2D gel electrophoresis. Computer-based analysis identified alterations in the expression of

19 of a total of 1,600 detectable proteins in GSNO-treated islets ($P < 0.01$). We conclude 1) that the expression of up to 42 proteins is altered by cytokine-induced or chemically generated NO in the precise experimental conditions chosen and 2) that the majority of proteins altered by prior treatment with IL-1 β may be the result of NO-independent IL-1 β -mediated regulation of gene expression. This study demonstrates that the combination of 2D gel electrophoresis and mass spectrometry is a powerful tool in the identification of β -cell proteins involved in the response to toxic mediators. *Diabetes* 49:1819–1829, 2000

The proinflammatory cytokine interleukin-1 β (IL-1 β) adversely affects the insulin-secretory response; the biosynthesis of proteins, DNA, and NAD; and even the viability of rat pancreatic islets of Langerhans (1–7) (reviewed by Mandrup-Poulsen [8]). Several of the actions of IL-1 β that lead to functional inhibition of insulin-containing cells have been suggested to require nitric oxide (NO) (2,3,9–11) (reviewed by Eizirik et al. [12]). NO is produced from arginine by the enzyme NO synthase (NOS). The calcium-independent inducible NOS (iNOS) or NOS2 is induced by IL-1 β (2,13–16) and other cytokines (17) in insulin-containing cell lines and rat islets. The use of arginine analogs such as N_G -monomethyl-L-arginine (NMMA) or N_G -nitro-L-arginine (L-NAME) has shown that NO from iNOS participates in the following actions of IL-1 β : inhibition of insulin secretion (2,3,18), decreased activity of aconitase and glucose oxidation (9,10), induction of DNA damage (19,20), and necrotic (21) and apoptotic cell death (5,6,22,23) in insulin-containing cell lines and rat islets.

NO-specific effects have been investigated in many cell types using chemically synthesized donors. Exposure of insulin-containing cell lines or islets of Langerhans to NO-releasing compounds, such as S-nitrosoglutathione (GSNO), 3-morpholiniosydnonimine (SIN-1), or Roussin's black salt, results in inhibition of glucose-induced insulin secretion, which is dose- and time-dependent (11,24–26). We have also found that NO donors decrease glucose oxidation (25) and redox function in insulin-containing cell lines and rat islets (6). NO donors have been reported to inhibit total protein synthesis in hepatocytes (27), vascular smooth muscle cells (28),

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2D, two-dimensional; ANOVA, analysis of variance; cGMP, cyclic GMP; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; GSNO, S-nitrosoglutathione; HBSS, HEPES-buffered saline solution; HSP, heat shock protein; IEF, isoelectric focusing; IL-1 β , interleukin-1 β ; iNOS, inducible NOS; irp94, ischemia-responsive protein 94 kDa; KRBH, Krebs-Ringer buffered HEPES; L-NAME, N_G -nitro-L-arginine; MALDI, matrix-assisted laser desorption/ionization; MnSOD, manganese superoxide dismutase; NEPHGE, nonequilibrium pH gradient electrophoresis; NMMA, N_G -monomethyl-L-arginine; NO, nitric oxide; NOS, NO synthase; PDI, protein disulfide isomerase; ROK, ribonucleoprotein K; SIN-1, 3-morpholiniosydnonimine.

and the insulin-containing cell line HIT-T15 (6). Furthermore, these donors caused necrotic cell death in rat islets in as little as 4 h when used at high doses (29). Low-dose NO donors induce apoptotic cell death in HIT-T15 cells and rat islets after as little as 24 h of exposure (5,6).

Treatment of islets with a low dose of IL-1 β (0.5 U/ml) for 24 h increases (pro)insulin and total protein biosynthesis (30). However, higher doses of IL-1 β (5 and 21 U/ml) decrease both (pro)insulin and total protein biosynthesis in insulin-containing cells (6,7,30,31). This inhibitory effect of IL-1 β on protein synthesis is reproduced by exposure of cells to NO donors (6) and is eliminated using the arginine analog NMMA (6,32). In rat islets, IL-1 β has been shown to increase the expression of proteins such as iNOS (14–16), heat shock protein (HSP) 70 (33–36), manganese superoxide dismutase (MnSOD) (36), and heme oxygenase (34,36,37). Upregulation of expression of some of these proteins by IL-1 β may be β -cell specific (36,38,39) and has recently been confirmed to occur in fluorescence-activated cell sorter (FACS)-purified rat β -cells (40). The action of IL-1 β on islets relies, to some extent, on *de novo* protein synthesis, because the inhibitor cycloheximide prevents IL-1 β suppression of insulin secretion and glucose oxidation (10,33,41,42). Two-dimensional (2D) gel electrophoresis of neonatal rat islet proteins, labeled with [³⁵S]methionine after exposure to IL-1 β for 24 h, has revealed alterations in the expression of 33 proteins when gel patterns of cytokine-treated and untreated islets were visually compared (43). After generation of a more sensitive computer-based database of >2,000 proteins synthesized *de novo* in cultured neonatal rat islets, the expression of 105 proteins has been found to be significantly altered by IL-1 β (44).

Our aim was to investigate whether treatment of neonatal rat islets with NO, derived from IL-1 β or a chemical donor, altered levels of expression of proteins by 2D gel electrophoresis. Two approaches were used: the first inhibited cytokine-induced NO production in cultured rat islets using the arginine analog L-NAME for 24 h before protein labeling; the second used the NO donor GSNO (6,24,25) for the same period of time as IL-1 β to reproduce its inhibitory effects and to potentially change rat islet protein expression.

RESEARCH DESIGN AND METHODS

Neonatal rat islet isolation and culture. Islets were isolated from the pancreases of 4- to 8-day-old male and female Wistar Furth rats (Charles River, Sulzfeldt, Germany) by collagenase digestion (Collagenase A; Boehringer Mannheim) followed by hand-picking. Islets were precultured in 60-mm tissue culture dishes (Nunc, Denmark) for 4–7 days at 37°C in atmospheric humidified air in 5 ml complete RPMI-1640 medium (Gibco, Paisley, U.K.) containing 11 mmol/l glucose, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Neonatal rat islet treatment before protein labeling. After preculture, islets were incubated for 24 h in RPMI-1640 medium prepared \pm L-arginine (1.15 mmol/l) and supplemented with 0.5% normal human serum. A total of 150 islets were cultured in a 300- μ l medium of arginine-containing RPMI \pm IL-1 β (150 pg/ml or 60 U/ml) (Novo Nordisk, Bagsvaerd, Denmark), arginine-free RPMI \pm IL-1 β , or arginine-free RPMI + L-NAME (1 mmol/l) (Sigma, St. Louis) \pm IL-1 β . In a separate series of experiments, a total of 150 islets in 300 μ l RPMI-1640 were exposed to three different NO donors, GEA 5583 (20–500 μ mol/l), GSNO (10–1,000 μ mol/l) (both from Alexis Corporation, Nottingham, U.K.), or SIN-1 (0.1–1,000 μ mol/l) (a gift from Cassella AG, Frankfurt, Germany). Of the three NO donors tested, only GSNO fulfilled the criteria for selection for 2D experiments, which was having normal islet integrity as seen under the dissection microscope and <50% inhibition of accumulated insulin release or [³⁵S]methionine incorporation. Therefore, only GSNO results are shown in the article.

Measurement of nitrite and cyclic GMP. Nitrite accumulation in conditioned media was determined by the Griess reaction (45) as described previously (2,46). A 200- μ l sample was removed from all wells after the 24 h incubation period for

measurement of medium nitrite accumulation. An equal volume of Griess reagent (one part 0.1% naphthylethylene diamine dihydrochloride and one part 1% sulphanilamide in 5% H₃PO₄ [Merck, Darmstadt, Germany]) was added to the samples in a microtiter plate and allowed to stand in the dark at room temperature for 10 min. Absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) plate reader (Nippon Inter Med, Tokyo) at 540 nm.

NO was also detected by changes in levels of cyclic GMP (cGMP) as described previously (11). Briefly, cyclic nucleotides were extracted from islets by adding 200 μ l of 50 mmol/l sodium acetate buffer (pH 4.7) and boiling for 3 min. Islet samples were centrifuged (1,000g), and the supernatants and pellets were stored at –20°C before assay. Protein in islet pellets was solubilized in 0.1 mmol/l NaOH and assayed using the Bradford method (47). cGMP in the supernatant was radioimmunoassayed (11,48), and results were expressed as picomoles of cGMP per microgram of islet protein.

Measurement of accumulated and stimulated insulin release. Of the islet treatment medium, 50 μ l was removed after 24 h incubation for measurement of accumulated insulin. Samples were assayed for insulin using an ELISA method (49). Islets were washed in low glucose-containing Krebs-Ringer buffered HEPES (KRBH) (1.67 mmol/l) once and incubated for 1 h at 37°C in 500 μ l of this buffer. The islets were transferred to high-glucose KRBH buffer (16.7 mmol/l) for 2 h at 37°C. Samples were removed for determination of basal and stimulated insulin release (ELISA) after each incubation period.

Labeling of islet proteins and 2D gel electrophoresis. After the 24-h treatment period, islets were washed twice in HEPES-buffered saline solution (HBSS) and labeled for 4 h in 200 μ l methionine-free Dulbecco's modified Eagle's medium (DMEM) (containing 19.4 mmol/l D-glucose and 0.4 mmol/l arginine) with 10% normal human serum dialyzed for amino acids and 200 μ Ci [³⁵S]methionine (previously freeze-dried for at least 4 h to remove 2-mercaptoethanol). After labeling, islets were washed three times in HBSS and were pelleted and frozen at –80°C. [³⁵S]methionine incorporation in the islet samples was determined, and 2D gel electrophoresis was performed as described previously (44).

Computerized analysis of the gels was performed using the BioImage program (version 4.6M; Genome Solutions) on a SunSparc workstation. Fluorographs were initially scanned and spots were identified and quantitated by the BioImage program. Each gel was then compared with the master gel of the 2D gel database of untreated rat islet proteins (44) and manually edited to ensure that those spots that had been originally found by the computer would be identified and quantified. This procedure was performed by the same observer (N.E.J.) using the BioImage program. The spots on each new gel were then matched by the BioImage program with those of the master gel, and the accuracy of the matching was checked and, where necessary, corrected by the same observer (N.E.J.). A sample of all editing and matching of spots was inspected by an independent observer (H.U.A.). Data from the program were analyzed in Microsoft Excel. The presence of duplicate spots in isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis (NEPHGE) gels was avoided by omitting overlapping spots present in either the basic portion of the IEF gels or in the acidic portion of the NEPHGE gels. The average percentage coefficient of variance value was calculated for each series of gels. Variance (mean \pm SD) of protein expression was increased from 29 \pm 19 and 26 \pm 10% in five control (database) gels to 33 \pm 20 and 43 \pm 26% in three IL-1 β gels and 63.3 \pm 26 and 61 \pm 37% in three IL-1 β +NAME-arginine gels for IEF and NEPHGE, respectively. In the second series of experiments, variance of protein expression was high in control (51 \pm 28 and 45 \pm 28%, IEF and NEPHGE, respectively, n = 3) and GSNO (44 \pm 29 and 41 \pm 31%, n = 3) gels. These variances were comparable with those reported previously for control and IL-1 β gels (44).

Identification of protein spots by mass spectrometry. The proteins were enzymatically digested in the gel as described (50,51) with minor modifications (52). The excised gel plugs were washed in 50 mmol/l NH₄HCO₃/acetonitrile (60/40) and dried by vacuum centrifugation. Modified porcine trypsin (12 ng/ μ l, Promega, sequencing grade) in digestion buffer (50 mmol/l NH₄HCO₃) was added to the dry gel pieces and incubated on ice for 1 h for reswelling. After removing the supernatant, 20–40 μ l digestion buffer was added and the digestion was continued at 37°C for 4–18 h. The peptides were extracted as described (51) and dried in a vacuum centrifuge. The residue was dissolved in 5% formic acid and analyzed by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. Delayed extraction MALDI mass spectra of the peptide mixtures resulting from in-gel digestion were acquired using a PerSeptive Biosystems Voyager Elite reflector time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). Samples were prepared using α -cyano-4-hydroxy cinnamic acid as a matrix. When appropriate, nitrocellulose was mixed with the matrix (53). Protein identification was performed by searching the peptide mass maps in a comprehensive nonredundant protein sequence database (European Bioinformatics Institute, Hinxton, U.K.) using the database search software PeptideSearch (54) further developed at EMBL (Heidelberg, Germany). The protein identifications were examined

TABLE 1
Incubation of islets in arginine-containing and arginine-free media

	Insulin (pmol · islet ⁻¹ · [24 h] ⁻¹)	Nitrite (nmol · islet ⁻¹ · [24 h] ⁻¹)
Arginine-containing medium		
Control	3.33 ± 0.33	2.35 ± 0.39
IL-1β	1.52 ± 0.24*	10.91 ± 4.52†
Arginine-free medium		
Control	2.61 ± 0.26	3.21 ± 1.34
IL-1β	2.67 ± 0.21‡	2.91 ± 1.18§
L-NAME	3.05 ± 0.32	3.1 ± 1.18
IL-1β + L-NAME	2.7 ± 0.21	3.45 ± 1.11§

Data are means ± SE from six experiments. * $P < 0.001$ and † $P < 0.05$ vs. control in arginine-containing medium; ‡ $P < 0.01$, § $P < 0.05$, and || $P < 0.005$ vs. IL-1β in arginine-containing medium.

using the second pass search feature of the software and critical evaluation of the peptide mass map as described by Jensen et al. (55).

Statistical analysis. Statistical analysis was performed using a two-sided unpaired Student's *t* test for insulin, nitrite, and cGMP measurements and a one-sided unpaired Student's *t* test for 2D gel analysis. Single-factor analysis of variance (ANOVA) was also performed. *P* values <0.05 were deemed to be significant in measurements of nitrite and insulin secretion. *P* values <0.05 and <0.01 have been used as specified in measurements of protein synthesis when analyzed by 2D gel electrophoresis.

RESULTS

The involvement of NO in IL-1β-induced changes in islet protein expression. Incubation of islets with IL-1β for 24 h in arginine-containing medium supplemented with

0.5% human serum resulted in a 4.6-fold increase in medium nitrite ($P < 0.05$ vs. control) and a >50% decrease in cumulative insulin release ($P < 0.01$ vs. control) (Table 1). Exposure of islets to IL-1β in arginine-free medium with or without L-NAME reversed both the rise in nitrite production ($P < 0.05$ vs. IL-1β) and the inhibition of insulin release ($P < 0.005$ vs. IL-1β) (Table 1). Islets were subjected to 4-h labeling with [³⁵S]methionine. Computerized analysis using the BioImage program revealed that, of the 105 protein spots showing altered expression with IL-1β treatment (44), the expression level of 23 was significantly different from the level seen by IL-1β alone (4 at $P < 0.01$ and 19 at $P < 0.05$

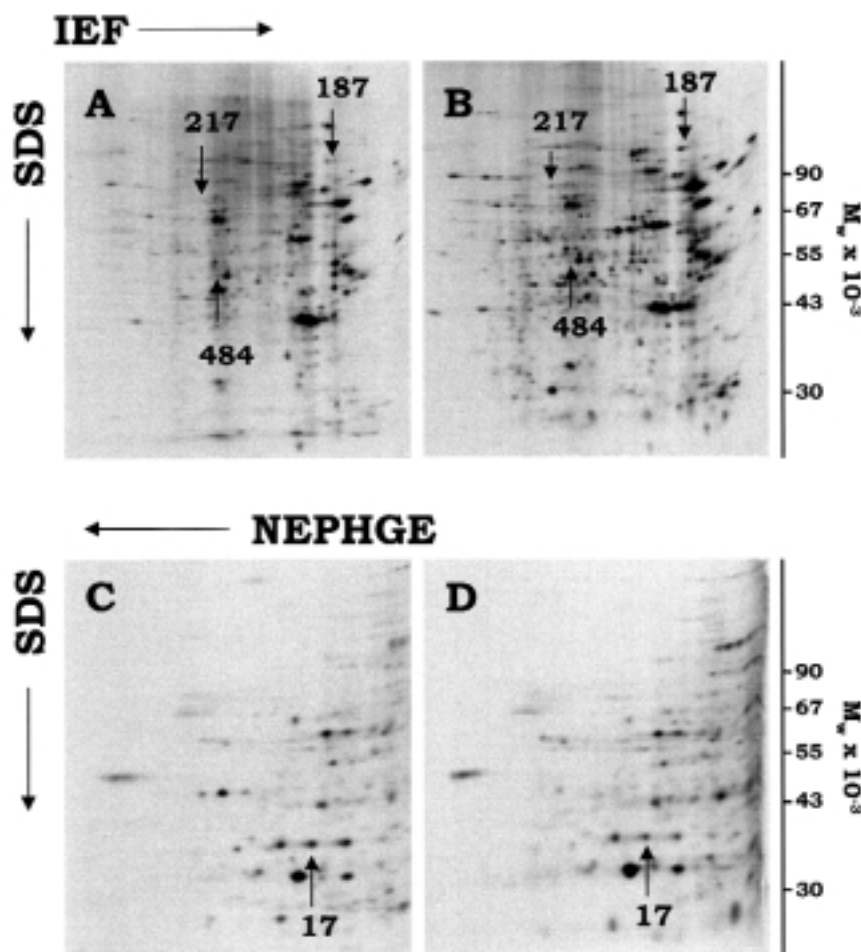


FIG. 1. 2D gel fluorographs of neonatal rat islets cultured with IL-1β (150 pg/ml) without arginine but with L-NAME (1.15 mmol/l) (A and C) or IL-1β with arginine (B and D) for 24 h followed by a 4-h labeling with [³⁵S]methionine. Gels shown are representative of three to four experiments. A and B represent IEF gels (pH 3.5–7.0), and C and D represent NEPHGE gels (pH 6.5–10.5). The numbers correspond to proteins identified in the database and in Table 2. For the sake of clarity, only proteins with $P < 0.01$ are shown. Arrows pointing upward and downward represent up- and downregulation, respectively.

TABLE 2
Identification by mass spectrometric analysis of islet proteins altered by IL-1 β plus L-NAME

Match no.	IL-1/C	IL-1 + NAME/C	IL-1 + NAME/IL-1	M _r kDa	pI	Identification	Gene Bank accession no.
IEF 10% Gels							
10	11.57	6.02	0.52	120.5	7.27		
28	2.65	1.64	0.62	65.8	5.06	Lamin B	D50080
						Heterogeneous nuclear ribonucleoprotein K/ROK	S41495
187*	6.19	0.93	0.15	152.1	4.95	Ischemia-responsive protein 94 kD	AAC27937
217*	4.73	1.23	0.26	91.3	6.12		
279	6.78	1.90	0.28	121.4	4.20		
330	2.74	0.97	0.36	68.8	5.35	Mortalin (GRP75)	P48721
340	0.32	1.02	3.18	71.3	5.20	Mortalin (GRP75)	P48721
354	6.23	1.81	0.29	70.4	4.30		
425	2.09	0.92	0.44	67.2	5.18	HSP71c (Heat shock cognate 71 kD protein)	P08109
484*	0.27	0.72	2.66	59.2	5.88	Protein disulfide isomerase (PDI) ER60 precursor (GRP58)	P11598
507	0.72	0.37	0.51	59.9	5.33	Mitochondrial matrix protein p1 precursor (HSP60)	P19227
831	0.53	0.25	0.48	34.6	4.76		
895	0.69	0.23	0.34	28.9	4.29	Tropomyosin NM4	L24777
908	0.27	0.12	0.43	25.8	6.28	Phospho-glycerate mutase (PGAM), brain form	P25113
1,196	9.55	3.92	0.41	143.1	5.41		
1,342	3.74	1.83	0.49	53.4	4.64		
NEPHGE 10% Gels							
17*†	5.09	8.50	1.67	40.0	8.29	GAPDH	P04797
156	3.22	1.09	0.34	55.6	8.71	Poly-pyrimidine tract-binding protein	Q00438
176	0.21	0.71	3.37	52.6	7.96	ATP synthase regulatory subunit (A)	P15999
211	0.28	0.07	0.26	47.9	7.28		
296	0.07	0.14	2.0	36.2	8.29	NADH-cytochrome B5 reductase	P20070
668	DN	DN	0.15	42.8	8.46	Fructose 1,6-biphosphate aldolase A*	P05065
674	DN	DN	2.4	132.5	8.42		

Ratios >1 represent an upregulation of protein expression. * $P < 0.01$; †spot intensities influenced also by L-NAME alone. DN, de novo.

[Fig. 1 A–D]) after inhibition of endogenous NO production. Of the four proteins changed at $P < 0.01$, three were acidic (from IEF gels [Fig. 1A and B]) and one was basic (NEPHGE gels [Figs. 1C and D]). In 16 of the 23 protein spots, L-NAME, as expected, either reduced the IL-1-induced upregulation or decreased IL-1-induced downregulation. In the case of the seven remaining protein spots, L-NAME accentuated IL-1-induced downregulation (IEF 507,831,895,908; NEPHGE 211) or increased IL-1-induced upregulation (NEPHGE 17,674) (Table 2); the latter may represent nonspecific effects of L-NAME (see below). Of the 23 protein spots, 14 were positively identified by mass spectrometric analysis (Table 2). In the absence of NO, the heat shock, ischemia-responsive protein 94 kDa (irp94) (Fig. 2A and B), and the unknown protein (Fig. 2C and D) were downregulated, whereas protein disulfide isomerase (PDI) (Fig. 2E and F) and GAPDH (Figs. 2G and H) were upregulated (Table 2). To determine any nonspecific effect of L-NAME treatment on protein expression, the expression of these four proteins was investigated on gels obtained from islets exposed to L-NAME in arginine-free medium. Only one of four proteins, GAPDH, was affected, and it showed

significant upregulation when islets were exposed to the arginine analog L-NAME alone in the absence of both IL-1 β and arginine (ratio 6.0, $P < 0.0001$ vs. untreated islets [database]). To ensure that NO production was not induced during the labeling period, measurements of nitrite and islet cGMP levels were taken after the 4-h labeling. Results showed that nitrite was not detectable in the media when islets previously exposed to IL-1 β in arginine-free medium that was supplemented with L-NAME for 24 h were labeled in arginine-containing medium for 4 h. Levels of cGMP in these islets were unchanged compared with those in control islets (1.98 ± 0.37 vs. 1.73 ± 0.36 fmol cGMP/ μ g protein, control vs. IL-1 β plus L-NAME, respectively).

NO donors and islet protein expression. The NO donor GSNO was used to examine the effect of NO on the synthesis of islet proteins. Neonatal rat islets were cultured with GSNO for 24 h, and culture medium was assayed for nitrite and insulin. For comparison, islets were also exposed to IL-1 β for 24 h; this exposure resulted in a rise in nitrite production (1.0 ± 0.03 vs. 10 ± 1.8 μ mol/l nitrite, control vs. IL-1 β , respectively, $P < 0.001$ vs. control). (Data were expressed in micromoles per liter so that the results were comparable with NO donor

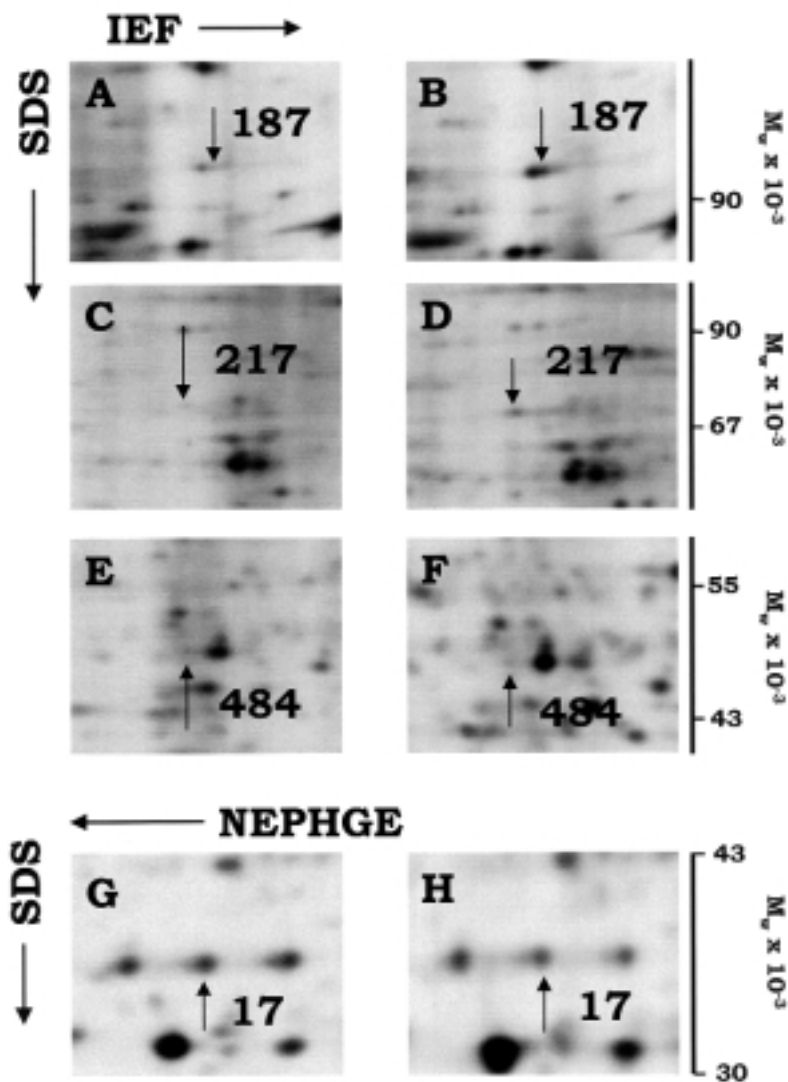


FIG. 2. Enlarged regions of NEPHGE and IEF gels of neonatal rat islets exposed to IL-1 β in the presence (B, D, F, and H) or absence of arginine with arginine analog addition (A, C, E, and G); the full gels are shown in Fig. 1. Proteins 187 (A) and 217 (C) (on IEF gels) are downregulated with inhibition of NO production compared with the presence of NO after IL-1 β treatment in arginine-containing medium (B and D). Protein spot 484 (NEPHGE) is upregulated with this treatment (E) compared with IL-1 β exposure in the presence of arginine (F). Protein spot 17 (NEPHGE) is upregulated after exposure to IL-1 β in the presence of an arginine analog (G) compared with treatment in the presence of arginine (H).

results.) Exposure of islets to increasing concentrations of GSNO caused a significant and expected dose-dependent increase in nitrite ($P < 0.001$ by single-factor ANOVA) (Fig. 3).

IL-1 β significantly inhibited insulin release into the treatment medium over a 24-h period (2.8 ± 0.3 and 0.8 ± 0.1 pmol

insulin \cdot islet $^{-1} \cdot [24 \text{ h}]^{-1}$, control and IL-1 β , respectively, $P < 0.001$ vs. controls). Exposure of islets to increasing concentrations of GSNO gave a significant dose-dependent inhibition of cumulative insulin release as determined by single-factor ANOVA ($P < 0.05$). GSNO (100 and 500 $\mu\text{mol/l}$) significantly inhibited insulin

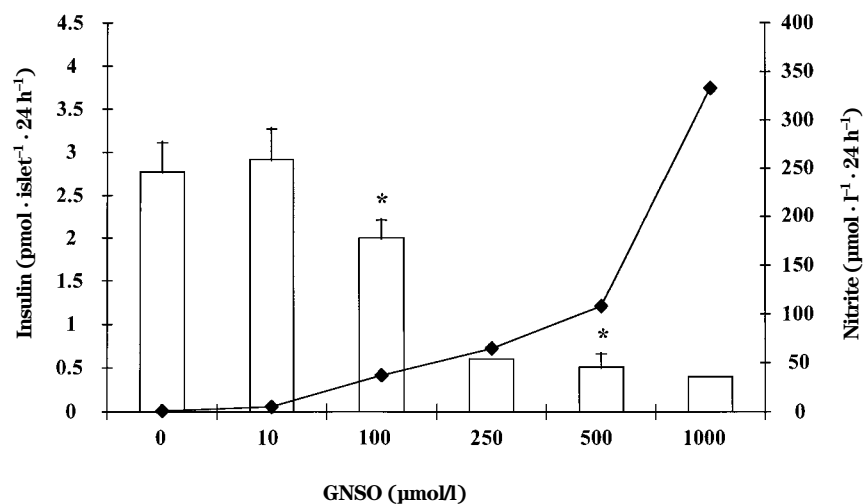


FIG. 3. Groups of islets were cultured with GSNO for 24 h. Medium was assayed for insulin (histogram) and nitrite (line). Data are presented as means \pm SE ($n = 5-12$ experiments); only one experiment was included for 250 and 1,000 $\mu\text{mol/l}$ GSNO. Student's two-sided t test was used for statistical analysis. Single-factor ANOVA confirmed a significant dose-dependent increase in medium nitrite ($P < 0.0001$) with all three donors and a dose-dependent inhibition of insulin release ($P < 0.05$) with GSNO treatment over the 24-h treatment period. * $P < 0.05$ vs. control without NO donor.

TABLE 3
GNSO-induced alterations in islet protein expression

Match no.	GSNO/ control ratio*	IL-1/control ratio†	M _r kDa	pI
IEF 10% Gels				
144	0.53	—	137.7	6.07
201	1.58	2.48	143.6	4.10
210	0.54	3.19	114.5	6.40
257	2.21	—	117.3	5.14
304	0.46	—	67.9	5.98
342	2.57	2.80	83.3	4.81
379	0.62	—	65.9	6.43
462	0.28	—	53.1	6.64
469	0.55	—	60.2	6.41
512	2.22	—	61.9	5.12
531	2.17	—	61.1	4.76
822	6.2	—	33.3	5.37
930	0.23	—	25.7	5.56
1,052	2.23	—	47.3	4.21
1,207	0.35	—	65.1	6.18
NEPHGE 10% Gels				
7	1.52	0.38	65.5	7.28
83	1.59	—	65.7	8.38
176	1.34	0.21	52.3	7.96
224	0.29	—	43.2	8.71

Data are the results of analyses of three experiments. *The average percent integrated optical density (IOD) of GNSO gels/average percent IOD of control gels; †the average percent IOD of IL-1 β gels/average percent IOD of database gels (44). A ratio higher than 1.0 indicates an upregulation of the spot compared with the control.

release into the culture medium (Student's *t* test, $P < 0.05$ vs. control) (Fig. 3). However, exposure of islets to GSNO at concentrations ≥ 150 $\mu\text{mol/l}$ resulted in visible islet disintegration as observed under a dissecting microscope. Consequently, 100 $\mu\text{mol/l}$ GSNO was used for the 2D gel experiments.

Computer-based analysis of IEF and NEPHGE gels from GSNO-treated islet samples showed that there were significant alterations in the expression levels of 15 acidic protein spots (IEF) and 4 basic protein spots (NEPHGE) when GSNO gels were compared with control gels (Table 3). Seven IEF protein spots were found to be upregulated and eight were downregulated with respect to control (Fig. 4A and B). Of the NEPHGE spots, three were upregulated and one was downregulated with respect to control (Fig. 4C and D). Specific protein spots are also identified in Fig. 5A–F. Of the total of 19 proteins that were altered with GSNO treatment, 5 had previously been found to be altered by IL-1 β treatment (44) (Table 3). Three of these five proteins were altered in a different direction to GSNO treatment, whereas the other two were upregulated by both GSNO and IL-1 β treatment. Two of these protein spots (NEPHGE gel spots 176 and 201), which were upregulated by GSNO treatment, were positively identified by mass spectrometric analysis as the α -chain of ATP-synthase and calnexin precursor, respectively.

DISCUSSION

In this study, we report the effects of IL-1 β - and chemically generated NO on the expression of neonatal rat islet proteins monitored by [³⁵S]methionine incorporation and 2D gel electrophoresis. The data are therefore compatible with those

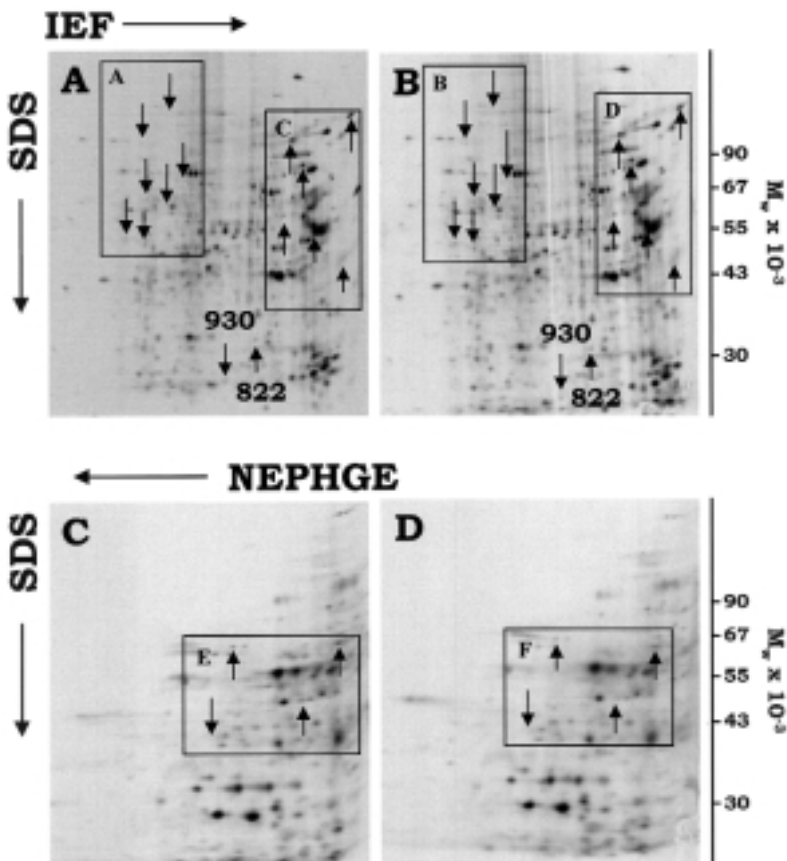


FIG. 4. Two-dimensional gel fluorographs of neonatal rat islets cultured with 100 $\mu\text{mol/l}$ GSNO for 24 h followed by a 4-h labeling with [³⁵S]methionine. Gels are representative of three experiments. Gels from GSNO-exposed islet samples are shown in A (IEF, pH 6.5–10.5) and C (NEPHGE, pH 3.5–7). Gels from control islet samples are represented in B (IEF) and D (NEPHGE). Numbers correspond to proteins in Table 3 with upregulated spots represented by arrows pointing upward and downregulated spots represented by arrows pointing downward. Boxed areas represent regions that have been enlarged in Fig. 5.

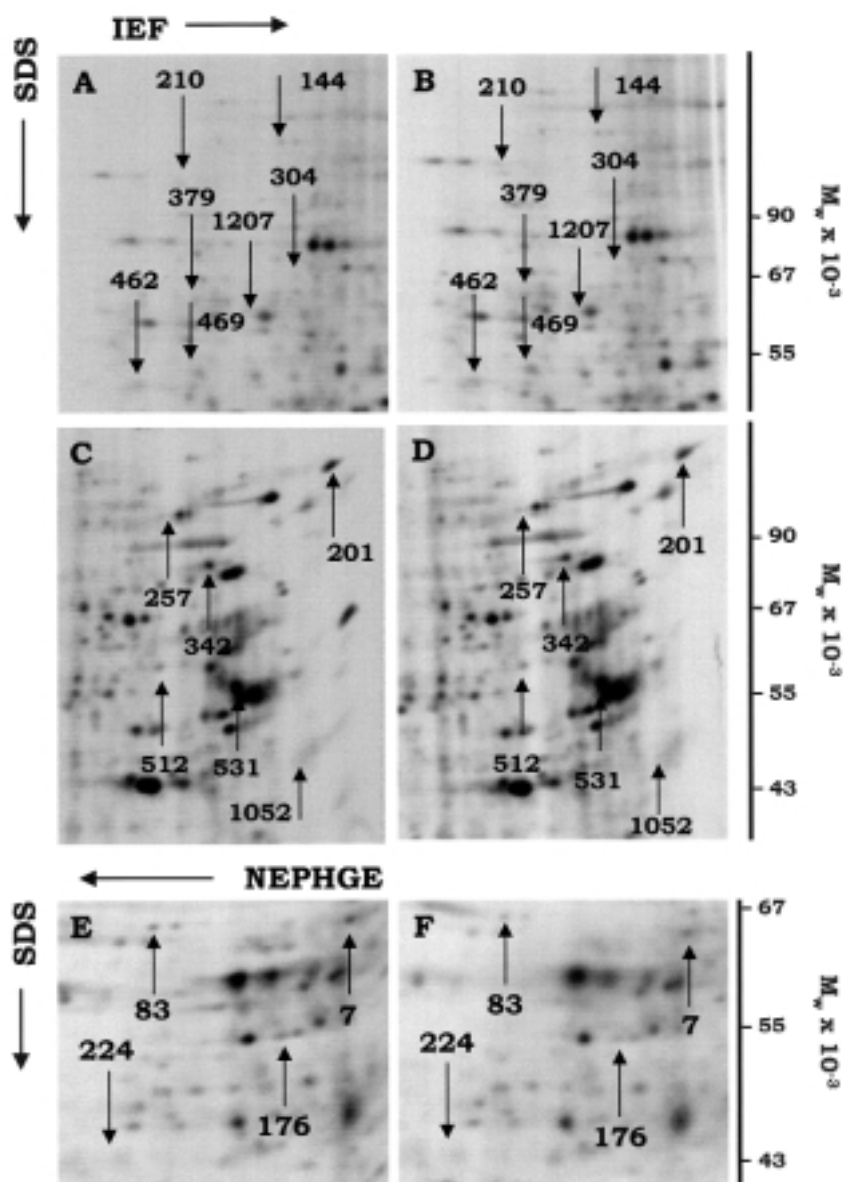


FIG. 5. Enlarged regions of NEPHGE and IEF gels of neonatal rat islets exposed to 100 $\mu\text{mol/l}$ GSNO for 24 h and control islets; the full gels are shown in Fig. 4. GSNO samples are represented in A, C, and E. Gels from control islet samples are represented in B, D, and F. The numbers correspond to proteins identified in the neonatal rat islet database and Table 3. Arrows pointing upward or downward represent up- or down-regulation, respectively.

held in the protein database (44). We compared the responsiveness of adult and neonatal rat islets to IL-1 in an earlier article (56) and found no difference, but we cannot exclude the possibility that the protein pattern of the two may be different. Preliminary studies show remarkable similarities between the protein expression of RIN cells and human islets (data not shown).

The computer-based analysis was carried out on 2D gel-separated islet proteins labeled for 4 h after earlier islet exposure to IL-1 for 24 h in the presence of either arginine or its analog, NAME. The expression levels of 23 of the 105 protein spots, known to be altered by IL-1 plus arginine, were significantly changed by IL-1 plus NAME ($P < 0.01$ [$n = 4$] and $P < 0.05$ [$n = 19$]). Of these 23 protein spots, 16 were increased, 7 were decreased, and 14 have been positively identified by mass spectrometric analysis and named for the first time here. Some increases in protein expression could be due to non-specific effects of L-NAME or to the existence of NO-dependent negative feedback on these proteins as previously suggested for iNOS (57) or the interleukin-1 converting enzyme (58).

Despite some experimental limitations, the combination of high-resolution 2D gel technology and mass spectrometry seems to be the method of choice. Messenger RNA differential display techniques have been applied to various target tissues, including islet tissue, in attempts to identify differences in specific mRNA expression and genes of relevance for type 1 diabetes (59,60). However, studies using mRNA differential display report primarily on de novo expressed transcripts and, to a lesser extent, on differential expression (61). Importantly, changes in mRNA levels may not always result in parallel alteration in protein levels. Finally, in contrast to mRNA (cDNA) expression studies, protein expression studies allow assessment of posttranslational modifications, which are often necessary for protein function and may also be of relevance in pathogenicity.

The proteins that are identified here for the first time, which were subject to the most pronounced alteration by cytokine treatment, merit attention. IL-1 β -increased expression of lamin B and/or ribonucleoprotein K (ROK) (both identified from database match number 28) was NAME reversible in

islets. Lamin B is primarily a structural nuclear-matrix protein that underlies the nuclear envelope; it has been studied in conjunction with induced apoptosis or necrosis (62), including that induced by 2D gel electrophoresis (63) in nonislet cells. ROK recruits molecular partners involved in transcriptional regulation and RNA processing (64).

Previous studies have shown that expression of islet HSP70, as determined by Western blotting, is increased upon IL-1 β treatment (34–36,65) and is prevented by coinubation with the arginine analog NMMA (65). These observations are in agreement with our findings that the IL-1 β -induced increases in the different heat shock proteins irp94, a presumed rat homolog of the mouse, human apg-2, a member of the HSP110 family (66) (match number 187), and the novel heat shock cognate 71 kDa protein (HSP71c, match number 425) were downregulated when NO production was inhibited (Fig. 2C and D and Table 2). HSP induction by IL-1 β treatment is commonly considered to be cytoprotective; however, in an experimental HSP70 overexpression system, cells were rendered more susceptible to cytotoxic T-cell-mediated apoptosis (67). It is of interest that a third protein in the large heat-shock family, mortalin (GRP75, match number 330 and 340) (68), seems to be posttranslationally modified in response to cytokines and the NO inhibitor because the expression levels were changed in opposite directions in the two spots. GRP75 is a glucose-regulated stress-inducible mitochondrial chaperone protein, possibly involved in antigen-processing and cell mortality (68).

PDI (match number 484), which was upregulated by inhibition of NO formation, is present in the lumen of the endoplasmic reticulum (ER) of cells that are actively synthesizing disulfide-bonded cell surface and secretory proteins; there may be implications for insulin biosynthesis and secretion (69,70). Although little is known about islet expression of PDI, a pancreatic isoform of this protein has been identified (71). Recent studies have shown that NO can react with PDI to inhibit platelet aggregation (72) and that PDI is involved in the entry of NO into cells (73).

IL-1 β -induced changes in tropomyosin (match number 895) in islets were relatively minor. Tropomyosin is thought to exist in equilibrium between two states of F-actin and is one of several 2D gel-identified proteins upregulated by cytokine treatment of a renal carcinoma cell line (74) or studied in transgenic overexpressing mice (75) exhibiting cardiomyopathy.

The expression of GAPDH (match number 17) was upregulated by inhibition of IL-1 β -induced NO production ($P < 0.01$ vs. IL-1 plus arginine; $P < 0.001$ vs. untreated islets). The NAME-induced increase in GAPDH could be an effect of NAME not being related to NOS inhibition, a suggestion supported by a recent study in which a 24-h incubation of rat pancreatic islets with IL-1 did not affect the expression of GAPDH (61) and by the fact that GSNO-mediated NO production did not increase GAPDH expression in the present study (Table 3). Endothelial cell GAPDH mRNA levels were, however, increased as a result of cytokine-generated NO exposure, perhaps as a compensatory mechanism to maintain physiological GAPDH production or to protect cells against NO cytotoxic effects (76). It is also possible that NO may be involved in oxygen sensing by competition with O₂ for cytochrome oxidase (77). Higher concentrations of NO would lead to hypoxia and the compensatory increase in glycolytic enzymes (including GAPDH).

Poly-pyrimidine tract-binding protein (match number 156) is a 60-kDa ribonucleoprotein that binds pre-mRNAs and is speculated to have a role in their splicing (78). It has also been studied in initiation of translation of human rhinovirus-2 RNA (79). Its elevation by IL-1 β treatment and reversal of this elevation by inhibition of NO production are both very pronounced.

F-1,6-bisphosphate aldolase A (a glycolytic enzyme) and NADH-cytochrome B5 reductase (match numbers 668 and 296, respectively) exhibit modest changes after islet treatment with IL-1 β . In contrast, IL-1 β greatly decreased ATP synthase regulatory subunit A (match number 176), an effect that was NO mediated. A role for ATP synthase in glucose protection against apoptosis in neurons has been proposed (80). Previous extensive research on ATP synthase, which uses a transmembrane proton gradient to drive synthesis of cellular ATP, has been reviewed (81).

The effects of the NO donor GSNO on neonatal rat islet protein expression were similarly investigated using 2D gel electrophoresis and computer-based analysis of gels. The treatment conditions used in this study were chosen in order to reproduce as closely as possible the conditions used by Andersen et al. (43,44) in 2D experiments on islets exposed to IL-1 β . Kinetics of NO release from IL-1 β induction of iNOS and NO donors in this study are unlikely to be directly comparable. We have previously measured NO production from GSNO using the isolated NO probe (IS NO; World Precision Instruments, Stevenage, U.K.) and found NO from 100 μ mol/l GSNO to be detectable within 20 min and still detectable after 3–4 h (82).

The expression of 19 proteins from a total of 1,600 detectable proteins was significantly affected by GSNO ($P < 0.01$). Of these 19 proteins, 5 were also affected by IL-1 β treatment (44), and 2 of the 5 have been positively identified by mass spectrometry as ATP-synthase α -chain and calnexin precursor, match numbers 176 and 201, respectively. The upregulation of ATP synthase seen in this study may be a compensatory mechanism by the cell to restore ATP production after NO exposure, because NO decreases ATP levels, at least in some cell types (82). Calnexin-precursor upregulation by NO suggests a requirement for molecular chaperoning (83). Calnexin is present in the ER, is phosphorylated by ER-associated protein kinases and detects mutated or misfolded proteins that are retranslocated, degraded, or secreted. However, in damaged cells, it has also been suggested to regulate expression of target molecules necessary for the binding of natural killer cells (84).

Previous studies have shown that exposure of insulin-containing cell lines, rat islets or human β -cells to chemically generated NO has functional effects that are in line with those of IL-1 β , including inhibition of insulin secretion (11,24–26), decreased redox function and cellular protein synthesis (6), induction of Fas expression (85), apoptosis (5,6), and necrosis (29). As the concentration of GSNO used here is substantially lower than that used in previous studies (6,24,25), it is possible that the changes in islet protein synthesis may relate to effects of GSNO on insulin secretion but not to effects on cell death. In addition, long-term exposure of islets to GSNO may have prevented detection of a number of early protein changes.

NO may have cytoprotective actions on cells depending on the concentration of NO applied and the cell type used. Exposure of cells to NO can be protective against tumor necrosis factor- α -induced cytotoxicity in endothelial cells

(86), ultraviolet A-induced apoptosis in human keratinocytes and endothelial cells (87), or apoptosis in hepatocytes (88). Suggestions of possible mechanisms involved in this protective effect of NO on cells have included cGMP-dependent upregulation of protective proteins such as heme oxygenase (86), induction of HSP70 expression (89), inhibition of caspases (88), or upregulation of bcl-2 expression with concomitant inhibition of Bax protein expression (87). Identification of NO-altered proteins may assist understanding of harmful and protective NO cellular actions.

In summary, we have used computer-based analysis of 2D gels to identify proteins having expressions specifically affected by inhibition of IL-1 β -induced NO or exposure to chemically generated NO. The outcome of these studies represents a further stage in our understanding of the role of NO in cytokine-mediated effects on β -cells and provides information for possible mechanisms of protection against harmful effects of NO.

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APPENDIX

We have recently identified the endoplasmic reticulum protein ERP29 precursor (ERP31) (acc. no. P52555) as part of spot 908 in addition to the phospho-glycerate mutase (PGAM). ERP31 is known to be induced by stress and may be involved in the processing of secretory proteins within the ER.

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